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FINAL REPORT

Study Title

Evaluation of a Test Article in the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay with Colony Size Evaluation in the Presence and Absence of Induced Rat Liver S-9 with a Confirmatory Study

Test Article

3-Nitro-1,2,4-Triazol-5-one (NTO)

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Laboratory Project I.D.

SITEK Study No.: 0985-2400

Study Initiation Date

August 26, 2008

Study Completion Date

November 17, 2008

Sponsor

US Army Center for Health Promotion and Preventive Medicine
Aberdeen Proving Ground, MD 21010

Sponsor's Study Coordinator

Gunda Reddy, Ph.D., DABT

Report Documentation Page

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Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
3-Nitro-1,2,4-triazol-5-one (NTO, 99.6% pure) was tested for its potentials to induce mutation at the thymidine kinase locus of L5178Y TK +/- mouse lymphoma cells in vitro with and without metabolic activation according to OECD TG 476 in compliance with Good Laboratory Practice. Cell cultures were treated with NTO at concentrations of 10, 50, 100, 250, 500, 1000, 2500, and 5000 ?g/mL with and without activation and were found negative in the mutation assay. A confirmatory mutation assay with 24 hrs treatment period and without activation was performed by using the same concentrations of NTO. These results were also negative.

15. SUBJECT TERMS

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STUDY DIRECTOR'S GLP COMPLIANCE STATEMENT

Study No.: 0985-2400
Sponsor's Test Article I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

The protocol for this study was designed to meet or exceed the ICH and OECD Guidelines specified in the following documents:

OECD Guideline for the Testing of Chemicals, No. 476, *In Vitro* Mammalian Cell Gene Mutation Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61(80):18198-18202, 1996.

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards except as indicated on the following page:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2004.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2004.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 Nohsan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998.

The strength and stability of the test article, dosing solutions and controls, under the experimental conditions, were not determined.

Signature: _____ 11-17-08

Paul E. Kirby, Ph.D.
Study Director

Date

QUALITY ASSURANCE UNIT'S STATEMENT

Study No.: 0985-2400
Sponsor's Test Article I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 08-26-08

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
09-04-08	Test Article Dilution	09-04-08	09-18-08
10-29-08	Workbook Audit	10-30-08	11-07-08
11-06-08	Draft Report Audit	11-07-08	11-07-08
11-17-08	Final Report Audit	11-17-08	11-17-08

Signature: _____ 11-17-08
Vian Lambert, B.S. Date
Manager, Quality Assurance Unit

STUDY DIRECTOR'S SIGNATURE PAGE

This study was performed under the supervision of Paul E. Kirby, Ph.D., Study Director for L5178Y TK$^{+/-}$ Mouse Lymphoma Mutation Assays, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Final Report for this study was written by Paul E. Kirby, Ph.D. and released on November 17, 2008.

Signature

Paul E. Kirby, Ph.D.
Study Director

11-17-08
Date

ABSTRACT

The test article, 3-Nitro-1,2,4-Triazol-5-one (NTO, 99.6% pure), was tested for its potential to induce mutations at the thymidine kinase locus of L5178Y $TK^{+/-}$ mouse lymphoma cells *in vitro*. The concentrations of test article tested with and without S-9 activation in the Range Finding Test were 0.1, 0.5, 1.0, 5.0, 10, 50, 100, 500, 1000, and 5000 µg/mL. The pH of the 5000 µg/mL solution was 3.09. It was adjusted to 6.8 with 1 N NaOH. Relative Suspension Growth (RSG, i.e., growth in culture after treatment compared to the growth in culture of the corresponding solvent control cultures) was used to measure toxicity. The RSG for cultures treated with NTO with and without S-9 activation indicated that NTO was relatively nontoxic. The cultures treated with and without S-9 activation with 5000 µg/mL had 57% and 86% RSG, respectively.

The Definitive Mutation Assay was performed using a 4-hour treatment period with and without S-9 activation at test article concentrations of 10, 50, 100, 250, 500, 1000, 2500, and 5000 µg/mL. Cultures treated with and without activation with concentrations of 250, 500, 1000, 2500, and 5000 µg/mL were cloned for mutant selection. All of the cultures, both with and without activation, had Mutant Frequencies (MF - mutants per 10^6 viable cells cloned) that were similar to the average MF of their concurrent solvent control cultures. The Relative Total Growth (RTG – the combination of suspension growth and clonal growth compared to that of the corresponding solvent control cultures) for the cloned cultures ranged from 72% to 132% for cultures treated without activation and from 18% to 73% for cultures treated in conjunction with exogenous activation. The results for a repeat of the with S-9 activation portion of the Definitive Mutation Assay also showed that the treated cultures all had MF that were similar to the average MF of the solvent control cultures. The RTG for these cultures ranged from 29% to 128%. Under the test conditions, the results of the Definitive Mutation Assay are considered negative.

Cultures were treated for approximately 24 hours without activation with concentrations of 10, 50, 100, 250, 500, 1000, 2500, and 5000 µg/mL for the Confirmatory Assay. The cultures treated with 250, 500, 1000, 2500, and 5000 µg/mL were cloned for mutant selection. All of the cultures had MF that were similar to the average MF of the solvent controls. The RTG for the cloned cultures ranged from 24% to 175%.

Under the test conditions, the results of the Definitive and Confirmatory Mutation Assays are considered negative.

TABLE OF CONTENTS

INTRODUCTION

The experimental portion of this study was conducted by Paul E. Kirby, Ph.D., Jian Song, Ph.D., Karen Shore, B.A., Melkie Lulie, M.S., Adrienne Parker, B.S., and Shashi Sharma, B.S. from August 27, 2008 to October 28, 2008, at SITEK Research Laboratories. The experimental procedures used to perform this study were essentially those of Donald C. Clive, et al. (1, 2) and they are described in detail in the protocol appended to this report.

The purpose of this study was to evaluate the test article, 3-Nitro-1,2,4-Triazol-5-one (NTO), for its potential to induce mutations at the thymidine kinase locus of L5178Y $TK^{+/-}$ mouse lymphoma cells.

In mammalian gene mutation assays treatment with the test chemical is carried out to induce a forward mutation at the specific locus. The cell cultures are incubated to allow phenotypic expression of any induced mutants. The cells are allowed to form macroscopic colonies in the presence of an agent that allows for only the growth of mutant cells.

The L5178Y mouse lymphoma clone used in this mutation assay is the $TK^{+/-}$3.7.2C strain which is heterozygous at the thymidine kinase (TK) locus. The TK enzyme is responsible for incorporating exogenous thymidine via a salvage pathway, into the cell in the form of thymidine monophosphate. Analogues of thymidine, such as trifluorothymidine (TFT), can also be phosphorylated by TK, which leads to cell toxicity. Forward mutation at this locus results in a loss of TK activity and subsequent resistance to TFT, which is used as the selective agent to kill wild type cells (6). $TK^{-/-}$ mutants are not killed by TFT and are able to survive due to the ability to synthesize purines de novo.

MATERIALS

INDICATOR CELLS

Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells, clone 3.7.2C, were originally obtained from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina, on October 17, 1984. The cells were subcultured, cleansed of TK-/- cells, and cryopreserved in a large number of ampules for L5178Y $TK^{+/-}$ Assays. The cells used for this study were from lot numbers 080808, 081508 and 091208.

Culture Conditions:

The L5178Y $TK^{+/-}$ mouse lymphoma cells were cultured in $RPMI_{10}$ [$RPMI_0$ Medium supplemented with 10% heat-inactivated horse serum] in 50 mL disposable centrifuge tubes at 37 ± 1°C on a roller drum rotating at approximately 25 rpm. Each culture was gassed with approximately 5% CO_2 and 95% air prior to placement on the roller drum. Each culture was sampled daily to determine cell concentrations, and if the cell concentrations were greater than $0.2x10^6$ cells/mL, the cultures were adjusted to $0.2x10^6$ cells/mL.

Stock Cultures

Stock cultures were grown in $RPMI_{10}$ in T-75 plastic tissue culture flasks on a shaker rotating at approximately 125 rpm. The cultures were monitored and adjusted when necessary to maintain them in log phase growth. The $TK^{+/-}$ cultures were cleansed of TK-/- cells by exposure to THMG (thymidine, hypoxanthine, methotrexate and glycine) for 24 hours and grown in THG (THMG without methotrexate) for an additional 24 hours prior to being cryopreserved.

CONTROL SUBSTANCES

Positive Controls

Methyl methanesulfonate (MMS), which induces mutations at the TK locus without metabolic activation, was used for the positive control for the without activation portion of the assay. The source and lot number of the MMS used in this study is given below:

MMS

Source: Sigma Chemical Company Lot No.: 123K3690

CAS Registry No.: 66-27-3 Storage Conditions: Room Temperature

Expiration Date: 11-06-08

7, 12-Dimethylbenz(α)anthracene (DMBA), which causes mutations at the TK locus with metabolic activation, was in the Definitive Assay. The source and lot number of the DMBA used in this study are given below:

Source: Sigma Chemical Company Lot No.: 096K1881

CAS Registry No.: 57-97-6 Storage Conditions: 1-5°C

Expiration Date: 03-05-09

Solvent Controls

DMSO was used to dissolve the test article. Information about the DMSO is presented below:

Source: Sigma Chemical Company Lot Nos.: 056K0172

CAS Number: 67-68-5 Storage Conditions: Room Temperature

Grade: Certified Expiration Dates: 01-16-12

The positive control substance DMBA was dissolved in acetone to make the stock solutions. The source and lot number of the acetone batch used in this study are given below:

Source: Mallinckrodt Lot No.: 2440 T15B29

Grade: Certified Storage Conditions: Room Temperature

CAS Registry Number: 67-64-1 Expiration Date: 11-13-10

The positive control substance MMS was dissolved in deionized, distilled water (DDW) to make the stock solutions. The source and lot number of the DDW was not recorded in the study workbook.

TEST ARTICLE

1. Name/Label ID: 3-Nitro-1,2,4-Triazol-5-one (NTO)
2. Batch/Lot No.: BAE 07B 305-001
3. Physical Appearance: White powder
4. Date Received: July 23, 2008
5. Storage Conditions: Refrigerated (1° to 5° C)
6. Purity Information: 99.6%
7. Correction Factor: None
8. Expiration Date: Not Provided

EXPERIMENTAL PROCEDURES

DOCUMENTATION

The materials, experimental procedures used in the performance of the study, experimental results and methods used in the evaluation of the results were documented in the study workbook.

DETERMINATION OF TEST ARTICLE SOLUBILITY

A solubility test was performed using water and DMSO. A small aliquot of the test article was dispensed into a glass test tube and water or DMSO was added. The test article had limited solubility in water; approximately 14 mg/mL. The test article was soluble at about 500 mg/mL in DMSO.

PREPARATION OF TEST CULTURES

Range Finding Test

The L5178Y $TK^{+/-}$ stock cultures were maintained in log phase growth until used in the study. A pool of cells was prepared from the stock cultures at a concentration of $1.0x10^6$ cells/mL in 50% conditioned $RPMI_{10}$ and 50% fresh $RPMI_0$ (RPMI 1640 medium, no serum added, containing 0.1% pluronics and 0.011% sodium pyruvate). Six (6.0) mL of the cell preparation was dispensed into 50-mL disposable centrifuge tubes resulting in $6x10^6$ cells/tube. Each tube was gassed with approximately 5% CO_2 and 95% air, sealed, and placed on a shaker to keep the cells suspended until treatment.

Mutation Assay

The cells were prepared for the Definitive Assay as described above for the Range Finding Test.

PREPARATION OF METABOLIC ACTIVATION SYSTEM

The metabolic activation system consisted of Aroclor 1254-induced rat liver homogenate (S-9 fraction) and the cofactor pool. The S-9 fraction was prepared in 0.25 M sucrose from Aroclor 1254-induced, male, Sprague-Dawley rats.

Immediately prior to treatment, the S-9 fraction was mixed with the cofactor pool to obtain the S-9 cofactor mixture, which was kept in wet ice until used. The S-9 cofactor mixture was prepared in the following proportion per mL of S-9 mix:

6.0 mg	NADP
11.25 mg	DL-Isocitric Acid
0.25 mL	S-9 Homogenate
0.75 mL	$RPMI_0$ Medium

All of the ingredients, except the S-9 homogenate, were mixed until they were dissolved. The pH of the solution was adjusted to approximately 6.8 by adding 1N NaOH until the color of the solution returned to its original media color. The solution was filtered by passing it through a 0.45 µm filter. The mixture was maintained on ice until it was used. At that time, the S-9 homogenate was added. In the Range Finding Test and the Definitive Assay, the S-9 mix was diluted 1:1 with serum free media before being used in the experiment.

The following are the details regarding the batches of S-9 used in this experiment:

Source:	Molecular Toxicology, Inc.
S-9 Batch Used:	2188 and 2220
Suspending Media:	0.25 M Sucrose
Protein content of the S-9:	35.8 and 40.1 mg/mL
Expiration Date:	10-03-09 and 12-05-09
Storage Conditions:	≤ - 70°C

Further information about the S-9 batches used is provided in Appendix V.

TEST SYSTEM IDENTIFICATION

All test cultures were labeled using an indelible ink pen with a code system, which clearly identified SITEK's Study No., experiment number, activation system, test article or control, and concentration.

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

For the Range Finding Test and the Definitive and Confirmatory Mutation Assays the test article was dissolved and diluted in DMSO in glass tubes. All the test article and control substance preparations and treatments were done under UV filtered lights to avoid possible problems of photoinactivation. The concentration and stability of the test article under experimental conditions was not determined.

RANGE FINDING TEST

In order to determine the test article concentrations that would produce cytotoxicity, a Range Finding Test was performed with and without activation at test article concentrations of 0.1, 0.5, 1.0, 5.0, 10, 50, 100, 500, 1000, and 5000 µg/mL.

Treatment was performed by adding 4.0 mL of $RPMI_0$ (serum-free RPMI media) or 4.0 mL of S-9 mix to the test cultures containing $6x10^6$ cells in 6.0 mL of medium and then adding 100 µL of each of the test article concentrations.

The solvent control was run simultaneously with the treated cultures in each system. The solvent control cultures received 100 µL of DMSO. The cultures were gassed with approximately 5% CO_2 and 95% air and incubated at 37 ± 1°C on a roller drum apparatus rotating at 25 ± 2 rpm.

After a 4-hour exposure period, the cells were pelleted by centrifugation at approximately 1000 rpm for 10 minutes, and the test article was removed by decanting the supernatant. Two rinses in 10 mL of $RPMI_{10}$ were performed, after which the cells were resuspended in 30 mL of $RPMI_{10}$, gassed with approximately 5% CO_2 and 95% air, and incubated at 37 ± 1°C on a roller drum rotating at 25 ± 2 rpm.

Approximately 20 hours and 44 hours post treatment, 1.0 mL samples were removed from each culture to determine the cell population density of each. The 1.0 mL sample was placed in a vial containing 19 mL of 0.1% trypsin in phosphate buffered saline. The vials were incubated for 10 minutes at 37 ± 1°C. Three counts were made using a Coulter Counter, and the average count was used to determine the concentration of cells per mL for each culture. After the determination of cell numbers at 20 hours post treatment, each culture having greater than $0.2x10^6$ cells/mL was adjusted to $0.2x10^6$ cells/mL. This was performed by retaining the volume of culture that would result in a final concentration of $0.2x10^6$ cells/mL when fresh medium was added to it to yield a combined final volume of 30 mL. The cultures having less than $0.2x10^6$ cells/mL were not adjusted. Either the adjusted, $0.2x10^6$ cells/mL or the lower unadjusted number was used as the Day 1 adjusted cell concentration.

The Suspension Growth (SG) of each culture was determined using the following formula:

$$SG = \frac{\text{Day 1 Cell Conc.}}{0.2x10^6 \text{ Cells/mL}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

Calculations were performed using a validated, Excel spreadsheet program (2400A.xlw).

The Relative Suspension Growth (RSG) of each of the test article-treated cultures was determined by calculating its growth relative to the corresponding solvent control cultures' average SG.

$$RSG = \frac{\text{SG of Treated Culture}}{\text{Average SG of Solvent Controls}} \times 100$$

MUTATION ASSAYS

The Definitive Mutation Assay was performed using a 4-hour treatment period with and without activation at test article concentrations of 10, 50, 100, 250, 500, 1000, 2500 and 5000

μg/mL with and without activation. The Confirmatory Mutation Assay was performed using a 24-hour treatment period without activation at test article concentrations of 10, 50, 100, 250, 500, 1000, 2500 and 5000 μg/mL.

The test article was dissolved in DMSO and a serial dilution was performed to prepare the lower test article dosing solutions. The pH of highest stock solution was acidic and was adjusted to approximately pH 6.8. Cultures containing 6.0 mL of cells at a concentration of $1.0x10^6$ cells/mL were prepared in conditioned $RPMI_{10}$ (supernatant from the stock cultures). Immediately prior to adding the appropriate test article dosing solution, 4.0 mL of either $RPMI_0$ medium or 4.0 mL of S-9 mix was added to each culture, depending on whether or not it was to be treated in conjunction with the exogenous metabolic activation system. 100 μL of each test article dosing solution was added to the appropriately labeled culture. The final volume at the end of the treatment was approximately 10 mL in each culture tube.

Immediately after addition of the test article, the culture was gassed with approximately 5% CO_2 and 95% air. After all of the cultures had been treated, they were placed on a roller drum apparatus and rotated at a speed of 25 ± 2 rpm in an environment of 37 ± 1°C. Two solvent control cultures were included in each treatment group, i.e., with activation and without activation. The solvent control cultures received 100 μL of DMSO.

In addition, two sets of cultures were treated with positive control chemicals. As indicated earlier, MMS was the positive control for the non-activated portion of the assay, and DMBA was the positive control for the S-9 activated portion of the assay. Prior to treatment, the positive controls, MMS and DMBA, and their respective solvent controls, deionized, distilled water and Acetone, received either 4.0 mL of serum-free medium or 4.0 mL of exogenous metabolic activation mixture in each tube just prior to treatment with 100 μL of the appropriate solution.

After a 4-hour exposure period, the cells were pelleted by centrifugation at approximately 1000 rpm for 10 minutes, and the test article was removed by pouring off the supernatant. Two rinses in 10 mL of $RPMI_{10}$ were performed. The final rinse was followed by resuspension in 30 mL of $RPMI_{10}$, gassing with approximately 5% CO_2 and 95% air, and incubation at 37 ± 1°C on a roller drum apparatus set at 25 rpm.

Approximately 20 hours and 44 hours post treatment, 1.0 mL samples were removed from each culture to determine the cell population density of each. The 1.0 mL sample was placed in a vial containing 19 mL of 0.1% trypsin. The vials were incubated for 10 minutes at 37 ± 1°C, after which they were placed on an automatic cell counter. Three coincidence-corrected counts were made, and the average count was used to determine the concentration of cells per mL for each culture. After the determination of cell numbers, each culture having a population greater than $0.2x10^6$ cells/mL was adjusted to $0.2x10^6$ cells/mL. The cultures having less than $0.2x10^6$ cells/mL were not adjusted. Either the adjusted, $0.2x10^6$ cells/mL or the lower unadjusted number was used as the Day 1 adjusted cell concentration. At the 20-hour point, the final volume after adjustment was 30 mL. At the 44-hour point, the final volume was 15 mL.

The Confirmatory Mutation Assay was conducted as above, except the cells were exposed for 24 hours and the cultures were adjusted at the end of treatment, and the following two days with cloning after the last adjustment.

Cloning for Mutants and Viability

After the expression period in the Mutation Assays cultures were selected for cloning based on their SG. Since the test article was nontoxic, the cultures treated with the five highest concentrations were selected for cloning.

For each culture selected for cloning, 200 mL of cloning medium (CM) was prepared. The CM was made by combining the following ingredients in the indicated proportions for each 100 mL of CM:

$RPMI_0$	70.75 mL
Horse Serum	20.0 mL
Sodium Pyruvate (0.022 gm/mL)	0.5 mL
Purified Agar (4% Solution)	8.75 mL

For each culture selected for cloning, 100 mL of CM was dispensed into a flask designated for the addition of the restrictive agent trifluorothymidine (TFT) (allowing the growth of TK-/- cells only) and 100 mL of CM was dispensed into a flask designated as a Viable Count (VC) flask. The CM in the VC flask was used to culture an aliquot of cells from each culture cloned to approximate the percentage of viable cells in each culture.

The cloning process was as follows:

1. Each TFT and VC flask received 100 mL of CM, and each was placed in a shaker incubator set at approximately 125 rpm and 37 ± 1°C.

2. The cultures designated for cloning were centrifuged at approximately 1000 rpm for 10 minutes. Twelve (12) mL of the supernatant was aspirated and discarded. The cells were resuspended in the remaining volume of supernatant and then added to the appropriate TFT flask. Each TFT flask contained $3x10^6$ cells. Each flask was replaced on the shaker incubator (125 rpm, 37 ± 1°C).

3. After each TFT flask had shaken for at least 15 minutes, a 1.0 mL aliquot was removed from each, a 1:10 and a 1:5 serial dilution (1:50 total dilution) was performed, and a 1.0 mL aliquot of the last dilution was added to the appropriate VC flask. Each VC flask contained approximately 600 cells.

4. After the completion of cell addition to each VC flask, an aliquot of TFT was added to each TFT flask. The concentration of TFT in the culture was approximately 3.0 µg/mL. Both TFT and VC flasks were replaced on the shaker incubator (125 ± 2 rpm, 37 ± 1°C).

5. After at least 15 minutes of mixing, the contents of each flask were dispensed in equal aliquots onto three plates. The plates were chilled for 20 minutes in a refrigerator (1-5°C) and then placed in an incubator at 37 ± 1°C in an atmosphere of approximately 5% CO_2 and 95% air for 12 days.

6. 3-4 days after cloning, the plates were carefully observed for signs of contamination. Any contaminated plate was discarded and the information was recorded in the study notebook.

Enumeration of Colonies

After an 11 to 12-day incubation period, the number of colonies per TFT and VC plate was determined by counting them with an ARTEK 880 Colony Counter. The raw counts were increased by a correction factor determined for SITEK's colony counter. The correction factor used was as follows:

$$\text{Adjusted Count} = \frac{(\text{Machine Count} - 0.649)}{0.8423}$$

Determination of Mutant Frequency and Induced Mutant Frequency

The Mutant Frequency (MF) of each culture that was successfully cloned was determined as a function of viable cells forming colonies. The calculation was performed as follows:

$$\text{MF}/10^6 \text{ Viable Cells} = \frac{\text{Average No. Mutants Per (RM)}^1 \text{ Plate}}{\text{Average No. of Colonies in the Corresponding VC Plates}} \times 200$$

The Induced Mutant Frequency (IMF) was calculated by using the following formula:

IMF = MF of Treated Cultures - Average MF of Solvent Control Cultures

Determination of Suspension Growth, Relative Suspension Growth, Relative Cloning Efficiency, Total Growth and Relative Total Growth

Relative Suspension Growth

The SG and RSG of each culture were determined by performing the calculations described earlier.

Relative Cloning Efficiency

The Relative Cloning Efficiency (RCE) was determined for each culture by using the following formula:

[1] RM refers to restrictive medium, i.e., plates containing medium with TFT.

$$RCE = \frac{\text{Average VC Count of Treated Culture}}{\text{Average VC Count of Solvent Controls}} \times 100$$

Total Growth

The Total Growth (TG) of a culture was calculated as follows:

$$TG = \frac{RSG \times RCE}{100}$$

The TG was calculated for each test article-treated culture that was successfully cloned. All of the calculations were performed using a validated, Excel spreadsheet program 2400B1.xls. The Relative Total Growth (RTG) was calculated by using the following formula:

$$RTG = \frac{\text{TG of Treated Culture}}{\text{Average TG of Solvent Controls}} \times 100$$

Sizing of Colonies

Colony sizes were determined by varying the size-setting potentiometer on an ARTEK 880 Colony Counter. Counts were first made with the potentiometer set so that all of the colonies would be counted. Subsequent counts were made with potentiometer settings adjusted so that approximately fifteen size groups were established for each plate.

The number of colonies in each size group was calculated and plotted with the increasing colony size with validated, Excel spreadsheet programs (SOLNASIZ.xlw, SOLS9SIZ.xlw, MMSSIZE.xlw and DMBASIZE.xlw). These graphs can be found in Appendix II.

CRITERIA FOR A VALID ASSAY

The following criteria were used in evaluating the acceptability of the assays.

Solvent Control Cultures

The results for the solvent control were considered acceptable if:

1. The average Cloning Efficiency of the solvent control cultures was 65% or higher.

2. The average MF of the solvent control cultures was between 35 to 140 per $1x10^6$ viable cells.

3. The suspension growth for the solvent controls was between 8 and 32 for the Definitive Assay and 23 to 180 for the Confirmatory Assay.

Positive Controls

The results for the positive control cultures were considered acceptable if:

1. At least one of the positive control-treated cultures had a MF that was three times or greater than the average MF of its solvent control cultures.

2. Their solvent controls had an average Cloning Efficiency of 65% or greater.

EVALUATION OF TEST RESULTS

The following criteria were used as guidelines in evaluating the results of the assays for a negative, positive or equivocal response. Since it is impossible to write criteria that would apply to every configuration of data generated by an assay, the Study Director was responsible for the ultimate decision in the evaluation of the results. No statistical methods were used in analyzing the data.

Criteria for a Positive Response

A response was considered positive if at least one culture had a MF that was two times or greater than the average MF of the corresponding solvent control cultures and the response was dose dependent.

Criteria for a Negative Response

A response was considered negative if:

1. All of the cultures exhibiting TG of approximately 10% and greater had MFs that were less than twice that of the mean MF of the corresponding solvent control cultures, and

2. There was no evidence of a dose-dependent response.

Criteria for an Equivocal Response

A response was considered equivocal if it did not fulfill the criteria of either a negative or a positive response, and/or the Study Director did not consider the response to be either positive or negative.

ARCHIVES

All of the raw data, documentation, protocol, protocol amendments, deviations and report, along with an electronic file containing the data tables and the final report of the study, are maintained for at least ten years at SITEK Research Laboratories' Archives at 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

RESULTS

SOLUBILITY TEST

The test article was soluble in water at approximately 14.3 mg/mL, and in DMSO at approximately 500 mg/mL.

RANGE FINDING TEST

The results for the Range Finding Test and Mutation Assays are presented in Appendix I.

A summary of the results of the Range Finding Tests is presented in Table 1. The concentrations of test article tested in the Range Finding Test were 0.1, 0.5, 1.0, 5.0, 10, 50, 100, 500, 1000, and 5000 µg/mL with and without S-9 activation. The test article was relatively nontoxic both with and without activation. The culture treated with 5000 µg/ml without activation had Relative Suspension Growth (RSG) of 86% and the culture treated with 5000 µg/mL with activation had 57% RSG.

DEFINITIVE MUTATION ASSAY

The Definitive Mutation Assay was performed using a 4-hour treatment period with and without activation at test article concentrations of 10, 50, 100, 250, 500, 1000, 2500, and 5000 µg/mL with and without activation. After the two-day expression period, cultures were selected for cloning based on their RSG. Since the test article was relatively nontoxic, the cultures treated with the five highest concentrations were cloned; 250, 500, 1000, 2500 and 5000 µg/mL.

The results of the Definitive Mutation Assay are presented in Tables 2-4 (Cloning Data) and Tables 5 and 6 (Total Growth Data). The results of a repeat assay[2] with activation are presented in Tables 7 and 8 (Cloning Data) and Tables 9 and 10 (Total Growth Data).

None of the cultures treated in the absence or presence of exogenous metabolic activation had Mutant Frequencies (MF) that were more than two-fold greater than the average MF of the corresponding solvent controls. The Relative Total Growth (RTG) for the cultures treated without activation ranged from 72% to 132%. The cultures treated in the presence of activation had RTG that ranged from 18% to 73% in the first trial, and from 29% to 128% in the second trial. Since the absolute number of mutant colonies were within the range of the corresponding solvent control values and there was no evidence of a dose response in relation to mutant frequency, these results are negative.

The positive control MMS produced a positive response without exogenous metabolic activation in the first trial and DMBA produced a positive response in the second trial. The

[2] The culture treated with the higher dose of DMBA was lost to contamination in the first trial, and the culture treated with the lower dose did not have a sufficient increase in mutant frequency.

solvent controls' MF values were within an acceptable range[3] of SITEK's historical solvent control values (Appendix IV). The size distribution for the cultures treated with the positive controls, MMS and DMBA, exhibited an acceptable positive response and colony size distribution.

CONFIRMATORY MUTATION ASSAY

The Confirmatory Mutation Assay was performed using a 24-hour treatment period without activation at test article concentrations of 10, 50, 100, 250, 500, 1000, 2500, and 5000 µg/mL with and without activation. After the two-day expression period, cultures treated with concentrations of 250, 500, 1000, 2500 and 5000 µg/mL were cloned for mutant selection.

The results of the Confirmatory Mutation Assay are presented in Tables 11 and 12 (Cloning Data) and Tables 13 and 14 (Total Growth Data).

As in the assay with the 4-hour exposure, all of the treated cultures had MF that were similar to that of the solvent control cultures. The RTGs for these cultures ranged from 24% to 175%.

The positive control, MMS, produced a positive response. The size distribution for the cultures treated with MMS exhibited an acceptable positive response and colony size distribution.

[3] The value for DMBA in the second trail was a little high. Please see protocol deviation number 1 for details.

CONCLUSIONS

The test article, 3-Nitro-1,2,4-Triazol-5-one (NTO), was tested in the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay both in the presence and absence of exogenous metabolic activation.

All of cloned cultures treated with NTO, up to the highest concentration tested of 5000 µg/mL[4], in the Definitive and Confirmatory Assays had Mutant Frequencies that were similar to that of their corresponding solvent control cultures. Therefore, under these test conditions, the test article, NTO, is negative in the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

[4] 5000 µg/mL is the highest recommended concentration by the ICH and OECD guidelines.

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APPENDIX I

SUMMARY TABLES

TABLE 1

L5178Y TK +/- MOUSE LYMPHOMA ASSAY

RANGE FINDING TEST

TRIAL NUMBER: A-1 TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol -5-one (NTO)

TEST DOSES IN: µg/mL SITEK STUDY NUMBER: 0985-2400

SOLVENT: DMSO

CONCEN-TRATION	CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	SUSPENSION GROWTH (SG)	RELATIVE SUSPENSION GROWTH
WITHOUT ACTIVATION				
SOLVENT A	0.642	0.603	9.7	AVERAGE SG
SOLVENT B	0.638	0.474	7.6	8.7
0.1	0.737	0.511	9.4	108%
0.5	0.702	0.491	8.6	99%
1.0	0.650	0.518	8.4	97%
5.0	0.634	0.485	7.7	89%
10	0.732	0.603	11.0	126%
50	0.780	0.480	9.4	108%
100	0.702	0.601	10.5	121%
500	0.705	0.451	7.9	91%
1000	0.833	0.443	9.2	106%
5000	0.680	0.444	7.5	86%
WITH S9 ACTIVATION				
SOLVENT A	0.560	0.790	11.1	AVERAGE SG
SOLVENT B	0.512	0.776	9.9	10.5
0.1	0.485	0.727	8.8	84%
0.5	0.496	0.741	9.2	88%
1.0	0.530	0.859	11.4	109%
5.0	0.433	0.846	9.2	88%
10	0.499	0.740	9.2	88%
50	0.389	0.797	7.8	74%
100	0.448	0.774	8.7	83%
500	0.383	0.779	7.5	71%
1000	0.382	0.784	7.5	71%
5000	0.282	0.847	6.0	57%

$$SG = \frac{\text{Day 1 Conc.}}{\text{2.0E+05 cells/mL}} \times \frac{\text{Day 2 Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

$$RSG = \frac{\text{SG of Treated Cultures}}{\text{Avg. of SG of Solvent Controls}} \times 100$$

Table verified by: QAU: Study Director:

TABLE 2

L5178Y TK+/- ASSAY - CLONING DATA

WITHOUT ACTIVATION

EXPERIMENT NO: B-1 (Definitive Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

TEST DOSES IN: µg/mL STUDY NUMBER: 0985-2400 SOLVENT: DMSO

TEST ARTICLE CONCENTRATION		COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE COLONIES/ VC PLATE	CLONING EFFI-CIENCY	MUTANT FREQUENCY (MF)/1.0E+06 CELLS	INDUCED MF/ 1.0E+06 CELLS	RELATIVE TOTAL GROWTH ***
SOLVENT A		99	82	93	91	139	171	190	167	83%	109	AVE. SOL-VENT MF	
SOLVENT B		61	68	62	64	158	175	188	174	87%	74	92	
250	A	139	137	138	138	263	211	221	232	116%	119	27	72%
250	B	91	108	106	102	189	199	176	188	94%	109	17	122%
500	A	88	82	93	88	262	209	203	225	112%	78	-14	99%
500	B	119	130	123	124	272	224	244	247	123%	100	8	120%
1000	A	131	119	126	125	301	250	272	274	137%	91	-1	118%
1000	B	127	86	116	110	266	218	265	250	125%	88	-4	132%
2500	A	99	91	131	107	247	226	233	235	118%	91	-1	109%
2500	B	123	119	165	136	276	322	328	309	154%	88	-4	106%
5000	A	113	100	95	103	220	249	208	226	113%	91	-1	97%
5000	B	124	120	143	129	249	216	367	277	139%	93	1	117%

*** = Data in this column is from Table 5.

Mutant Frequency (MF)/ 1.0E+06 Viable Cells = $\frac{\text{Average Number of Mutants Per (RM) Plate x 200}}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: QK

TABLE 3

L5178Y TK+/- ASSAY - CLONING DATA

WITH S-9 ACTIVATION

EXPERIMENT NO: B-1 (Definitive Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

TEST DOSES IN: µg/mL STUDY NUMBER: 0985-2400 SOLVENT: DMSO

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE COLONIES/ VC PLATE	CLONING EFFI-CIENCY	MUTANT FREQUENCY (MF)/1.0E+06 CELLS	INDUCED MF/ 1.0E+06 CELLS	RELATIVE TOTAL GROWTH ***
SOLVENT A	152	157	160	156	243	254	251	249	125%	125	AVE. SOL-VENT MF	
SOLVENT B	174	202	193	190	260	327	294	294	147%	129	127	
250 A	105	135	137	126	211	257	224	231	115%	109	-18	66%
250 B	162	158	174	165	258	231	235	241	121%	137	10	73%
500 A	146	156	155	152	196	218	243	219	110%	139	12	52%
500 B	205	230	176	204	-1	-1	-1	#N/A	#N/A	#N/A	#N/A	#N/A
1000 A	94	132	94	107	211	224	182	206	103%	104	-23	50%
1000 B	171	163	175	170	187	263	180	210	105%	162	35	61%
2500 A	133	144	122	133	196	262	205	221	111%	120	-7	58%
2500 B	132	165	129	142	251	266	143	220	110%	129	2	58%
5000 A	92	151	106	116	156	168	114	146	73%	159	32	18%
5000 B	103	119	104	109	243	211	170	208	104%	105	-22	29%

-1 = Plate lost to contamination.
#N/A = Not applicable
*** = Data in this column is from Table 5.

Mutant Frequency (MF)/1.0E+06 Viable Cells = $\frac{\text{Average Number of Mutants Per (RM) Plate x 200}}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: PK

TABLE 4

L5178Y TK+/- ASSAY - CLONING DATA

POSITIVE CONTROLS

EXPERIMENT NO.: B-1 (Definitive Mutation Assay)

TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

MMS DOSES IN: µg/ml
DMBA DOSES IN: µg/ml

STUDY NUMBER: 0985-2400

SOLVENT FOR MMS: Water
SOLVENT FOR DMBA: Acetone

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE COLONIES/ VC PLATE	CLONING EFFI-CIENCY	MUTANT FREQUENCY (MF)/1.0E+06 CELLS	INDUCED MF/ 1.0E+06 CELLS	RELATIVE TOTAL GROWTH ***
											AVE. SOL-	
DMSO A	104	137	136	126	258	300	294	284	142%	89	VENT MF	
DMSO B	125	126	107	119	270	291	304	288	144%	83	86	
MMS												
10	42	50	51	48	55	53	63	57	29%	168	82	15%
15	127	138	149	138	112	108	141	120	60%	230	144	26%
20	97	170	161	143	86	95	117	99	50%	289	203	18%
											AVE. SOL-	
ACETONE	196	195	169	187	139	171	190	167	83%	224	VENT MF	
ACETONE	131	154	148	144	158	175	188	174	87%	166	195	
DMBA												
2.5	163	173	173	170	211	221	190	207	104%	164	-31	51%
5.0	-1	-1	-1	#N/A	-1	-1	-1	#N/A	#N/A	#N/A	#N/A	#N/A

MMS = Methyl Methanesulfonate
DMBA = 7, 12 Dimethylbenz(a)anthracene

-1 = Culture lost due to contamination.
#NA = Not Applicable
*** = Data in this column is from Table 6.

Mutant Frequency (MF)/ 1.0E+06 Viable Cells = $\frac{\text{Average Number of Mutants Per (RM) Plate x 200}}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: PK

TABLE 5

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

EXPERIMENT NO.: B-1 (Definitive Mutation Assay) STUDY NUMBER: 0985-2400

TEST DOSES IN: µg/mL TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

SOLVENT: DMSO

T. A. CONCEN-TRATION		CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	SUSPEN-SION GROWTH (SG)	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
WITHOUT ACTIVATION								
SOLVENT A		0.630	0.663	10.4	SOLVENT AVERAGE SG 10.7	167	AVE. NO. COLONIES 170	
SOLVENT B		0.641	0.689	11.0		174		
250	A	0.631	0.360	5.7	53%	232	136%	72%
250	B	0.676	0.699	11.8	110%	188	111%	122%
500	A	0.616	0.518	8.0	75%	225	132%	99%
500	B	0.610	0.584	8.9	83%	247	145%	120%
1000	A	0.642	0.483	7.8	73%	274	161%	118%
1000	B	0.650	0.593	9.6	90%	250	147%	132%
2500	A	0.759	0.446	8.5	79%	235	138%	109%
2500	B	0.624	0.398	6.2	58%	309	182%	106%
5000	A	0.469	0.665	7.8	73%	226	133%	97%
5000	B	0.528	0.587	7.7	72%	277	163%	117%
WITH S-9 ACTIVATION								
SOLVENT A		0.481	0.691	8.3	SOLVENT AVERAGE SG 7.6	249	AVE. NO. COLONIES 272	
SOLVENT B		0.491	0.559	6.9		294		
250	A	0.262	0.903	5.9	78%	231	85%	66%
250	B	0.325	0.760	6.2	82%	241	89%	73%
500	A	0.261	0.757	4.9	64%	219	81%	52%
500	B	0.188	0.893	4.5	59%	#N/A	#N/A	#N/A
1000	A	0.251	0.799	5.0	66%	206	76%	50%
1000	B	0.282	0.856	6.0	79%	210	77%	61%
2500	A	0.244	0.894	5.5	72%	221	81%	58%
2500	B	0.228	0.952	5.4	71%	220	81%	58%
5000	A	0.086	0.518	2.6	34%	146	54%	18%
5000	B	0.095	0.571	2.9	38%	208	76%	29%

#N/A = Not applicable

Suspension Growth (SG) = $\frac{\text{Day 1 Cell Concentration x Day 2 Cell Concentration}}{\text{2.0E+05 Cells/ml x Day 1 Adjusted cell Concentration}}$

Relative Suspension Growth (RSG) = $\frac{\text{SG of Treated Culture x 100}}{\text{Average SG of Solvent Controls}}$

Relative Cloning Efficiency (RCE) = $\frac{\text{Average Viable Counts of Treated Culture x 100}}{\text{Average Viable Counts of Solvent Controls}}$

Total Growth = (RSG x RCE)/100

Table verified by: QAU: VL Study Director: PK

TABLE 6

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

EXPERIMENT: B-1 (Definitive Mutation Assay) STUDY NUMBER: 0985-2400

HYC DOSES IN: µg/mL
DMBA DOSES IN: µg/mL

TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

CONCEN-TRATION	CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	SUSPEN-SION GROWTH (SG)	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
				SOLVENT		AVE. NO.	
WATER A	0.609	0.556	8.5	AVERAGE SG 8.6	284	COLONIES 286	
WATER B	0.582	0.598	8.7		288		
MMS							
10	0.563	0.471	6.6	77%	57	20%	15%
15	0.546	0.400	5.6	63%	120	42%	26%
20	0.493	0.358	4.4	51%	99	35%	18%
				SOLVENT		AVE. NO.	
ACETONE A	0.465	1.230	14.3	AVERAGE SG 14.5	167	COLONIES 170	
ACETONE B	0.500	1.172	14.7		174		
DMBA							
2.5	0.232	1.042	6.0	42%	207	122%	51%
5.0	0.035	0.471	2.4	16%	#N/A	#N/A	#N/A

#NA = Not Applicable

Suspension Growth (SG) = $\frac{\text{Day 1 Cell Concentration x Day 2 Cell Concentration}}{\text{2.0E+05 Cells/ml x Day 1 Adjusted cell Concentration}}$

Relative Suspension Growth (RSG) = $\frac{\text{SG of Treated Culture x 100}}{\text{Average SG of Solvent Controls}}$

Relative Cloning Efficiency (RCE) = $\frac{\text{Average Viable Counts of Treated Culture x 100}}{\text{Average Viable Counts of Solvent Controls}}$

Total Growth = (RSG x RCE)/100

Table verified by: QAU: VL Study Director: GK

TABLE 7

L5178Y TK+/- ASSAY - CLONING DATA

WITH S-9 ACTIVATION

EXPERIMENT NO: B-6 (Repeat Definitive Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

TEST DOSES IN: µg/mL STUDY NUMBER: 0985-2400 SOLVENT: DMSO

TEST ARTICLE CONCENTRATION		COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE COLONIES/ VC PLATE	CLONING EFFI-CIENCY	MUTANT FREQUENCY (MF)/1.0E+06 CELLS	INDUCED MF/ 1.0E+06 CELLS	RELATIVE TOTAL GROWTH ***
SOLVENT	A	93	108	89	97	136	100	127	121	61%	160	AVE. SOL-VENT MF	
SOLVENT	B	89	100	-1	95	107	174	164	148	74%	128	144	
250	A	69	106	87	87	167	228	150	182	91%	96	-48	89%
250	B	49	142	92	94	257	203	119	193	97%	97	-47	102%
500	A	86	105	97	96	227	226	148	200	100%	96	-48	81%
500	B	86	114	131	110	252	220	104	192	96%	115	-29	111%
1000	A	91	122	123	112	243	266	149	219	110%	102	-42	128%
1000	B	110	152	145	136	157	219	218	198	99%	137	-7	126%
2500	A	95	138	132	122	139	216	194	183	92%	133	-11	86%
2500	B	97	127	138	121	158	200	197	185	93%	131	-13	103%
5000	A	133	190	161	161	245	252	250	249	125%	129	-15	29%
5000	B	152	117	158	142	212	213	218	214	107%	133	-11	33%

*** = Data in this column is from Table 9.

Mutant Frequency (MF)/1.0E+06 Viable Cells = $\frac{\text{Average Number of Mutants Per (RM) Plate x 200}}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: PK

TABLE 8

L5178Y TK+/- ASSAY - CLONING DATA

POSITIVE CONTROLS

EXPERIMENT NO.: B-6 (Repeat Definitive Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

DMBA DOSES IN: µg/ml STUDY NUMBER: 0985-2400 SOLVENT FOR DMBA: ACETONE

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE COLONIES/ VC PLATE	CLONING EFFI-CIENCY	MUTANT FREQUENCY (MF)/1.0E+06 CELLS	INDUCED MF/ 1.0E+06 CELLS	RELATIVE TOTAL GROWTH ***
ACETONE A	108	120	132	120	224	184	183	197	99%	122	AVE. SOL-VENT MF	
ACETONE B	116	133	-1	125	206	173	167	182	91%	137	130	
DMBA												
1.0	146	141	151	146	213	151	180	181	91%	161	31	85%
1.0	146	146	176	156	228	126	125	160	80%	195	65	70%
2.5	87	72	224	128	214	101	85	133	67%	192	62	40%
2.5	88	119	161	123	168	87	59	105	52%	234	104	25%
5.0	168	194	164	175	135	75	31	80	40%	438	308	7%
5.0	99	184	174	152	92	63	31	62	31%	490	360	5%

DMBA = 7, 12 Dimethylbenz(a)anthracene

-1 = Culture lost due to contamination.

*** = Data in this column is from Table 10.

Mutant Frequency (MF)/ 1.0E+06 Viable Cells = $\frac{\text{Average Number of Mutants Per (RM) Plate x 200}}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: GK

TABLE 9

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

EXPERIMENT NO.: B-6 (Repeat Definitive Mutation Assay) STUDY NUMBER: 0985-2400

TEST DOSES IN: µg/mL TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

SOLVENT: DMSO

T. A. CONCEN-TRATION		CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	SUSPEN-SION GROWTH (SG)	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
WITH S-9 ACTIVATION								
SOLVENT A		0.676	0.645	10.9	SOLVENT AVERAGE SG 10.7	121	AVE. NO. COLONIES 135	
SOLVENT B		0.674	0.619	10.4		148		
250	A	0.401	0.705	7.1	66%	182	135%	89%
250	B	0.438	0.691	7.6	71%	193	143%	102%
500	A	0.303	0.773	5.9	55%	200	148%	81%
500	B	0.423	0.781	8.3	78%	192	142%	111%
1000	A	0.473	0.720	8.5	79%	219	162%	128%
1000	B	0.540	0.680	9.2	86%	198	147%	126%
2500	A	0.350	0.762	6.7	63%	183	136%	86%
2500	B	0.453	0.703	8.0	75%	185	137%	103%
5000	A	0.091	0.341	1.7	16%	249	184%	29%
5000	B	0.118	0.445	2.2	21%	214	159%	33%

Suspension Growth (SG) = $\frac{\text{Day 1 Cell Concentration x Day 2 Cell Concentration}}{\text{2.0E+05 Cells/ml x Day 1 Adjusted cell Concentration}}$

Relative Suspension Growth (RSG) = $\frac{\text{SG of Treated Culture x 100}}{\text{Average SG of Solvent Controls}}$

Relative Cloning Efficiency (RCE) = $\frac{\text{Average Viable Counts of Treated Culture x 100}}{\text{Average Viable Counts of Solvent Controls}}$

Total Growth = (RSG x RCE)/100

Table verified by: QAU: VL Study Director: PK

TABLE 10

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

EXPERIMENT: B-6 (Repeat Definitive Mutation Assay) STUDY NUMBER: 0985-2400

DMBA DOSES IN: µg/mL TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

CONCEN-TRATION	CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	SUSPEN-SION GROWTH (SG)	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
ACETONE A	0.659	0.546	9.0	SOLVENT AVERAGE SG 9.3	197	AVE. NO. COLONIES 190	
ACETONE B	0.677	0.562	9.5		182		
DMBA							
1.0	0.587	0.562	8.3	89%	181	95%	85%
1.0	0.544	0.565	7.7	83%	160	84%	70%
2.5	0.295	0.718	5.3	57%	133	70%	40%
2.5	0.226	0.745	4.2	45%	105	55%	25%
5.0	0.086	0.290	1.5	16%	80	42%	7%
5.0	0.098	0.284	1.4	15%	62	33%	5%

Suspension Growth (SG) = $\frac{\text{Day 1 Cell Concentration x Day 2 Cell Concentration}}{\text{2.0E+05 Cells/ml x Day 1 Adjusted cell Concentration}}$

Relative Suspension Growth (RSG) = $\frac{\text{SG of Treated Culture x 100}}{\text{Average SG of Solvent Controls}}$

Relative Cloning Efficiency (RCE) = $\frac{\text{Average Viable Counts of Treated Culture x 100}}{\text{Average Viable Counts of Solvent Controls}}$

Total Growth = (RSG x RCE)/100

Table verified by: QAU: VL Study Director: QK

TABLE 11

L5178Y TK+/- ASSAY - CLONING DATA

WITHOUT ACTIVATION

EXPERIMENT NUMBER: B-2 (Confirmatory Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

TEST DOSE UNITS: µg/mL SITEK STUDY NUMBER: 0985-2400 SOLVENT: DMSO

	CONCENTRATION		COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE CLONING COLONIES/ VC PLATE	EFFI-CIENCY	MUTANT FREQUENCY MF/1.0E+0(CELLS	INDUCED MF/ 1.0E+06 CELLS	*** RELATIVE TOTAL GROWTH
	SOLVENT	A	-1	-1	-1	#N/A	-1	-1	-1	#N/A	#N/A	#N/A	AVG. SOLVENT MF	
	SOLVENT	B	130	129	142	134	211	201	206	206	103%	130	130	
1	250	A	113	88	106	102	232	244	247	241	121%	85	-45	115%
2	250	B	160	136	144	147	361	346	363	357	178%	82	-48	175%
3	500	A	92	97	89	93	137	136	173	149	74%	125	-5	79%
4	500	B	122	101	111	111	148	146	157	150	75%	148	18	79%
5	1000	A	-1	-1	-1	#N/A	281	323	226	277	138%	#N/A	#N/A	149%
6	1000	B	122	111	103	112	240	244	264	249	125%	90	-40	117%
7	2500	A	131	106	91	109	228	208	216	217	109%	100	-30	68%
8	2500	B	112	119	103	111	195	150	194	180	90%	123	-7	68%
9	5000	A	85	89	93	89	148	165	156	156	78%	114	-16	24%
10	5000	B	62	61	66	63	130	141	143	138	69%	91	-39	26%

*** = Data in this column is from Table 13.
-1 = Culture lost due to contamination.
#N/A = Not applicable.

Mutant Frequency (MF)/ 1.0E+06 Viable Counts = $\frac{\text{Average Number of Mutants per (RM) Plate x 200}}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: BK

TABLE 12

L5178Y TK+/- ASSAY - CLONING DATA

POSITIVE CONTROL

EXPERIMENT NUMBER: B-2 (Confirmatory Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

MMS DOSE UNITS: µg/mL SITEK STUDY NUMBER: 0985-2400 SOLVENT: DMSO

CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (TFT) 1	2	3	AVERAGE COLONIES/ TFT PLATE	COLONIES PER VIABLE COUNT (VC) PLATE 1	2	3	AVERAGE COLONIES/ VC PLATE	CLONING EFFI-CIENCY	MUTANT FREQUENCY MF/ 1.0E+06 CELLS	INDUCED MF/ 1.0E+06 CELLS	*** RELATIVE TOTAL GROWTH
WITHOUT ACTIVATION												
DMSO A	78	88	69	78	193	206	209	203	101%	77	AVERAGE SOLVENT MF	
DMSO B	88	88	91	89	162	167	157	162	81%	110	94	
MMS 15	146	107	116	123	44	36	49	43	22%	572	478	1%
MMS 20	16	9	10	12	0	2	2	1	1%	2400	2306	0%

*** = Data in this column is from Table 14.

MMS = Methyl Methanesulfonate

Mutant Frequency (MF)/ 1.0E+06 Viable Counts = $\frac{\text{Average Number of Mutants per (RM) Plate} \times 200}{\text{Average Number of Colonies in the Corresponding Viable Counts (VC) Plates}}$

Induced Mutant Frequency (IMF) = MF of Treated Cultures - Average MF of Solvent Control Cultures

Table verified by: QAU: VL Study Director: BY

TABLE 13

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

EXPERIMENT NUMBER: B-2 (Confirmatory Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

TEST DOSE UNITS: µg/mL SITEK STUDY NUMBER: 0985-2400

SOLVENT: DMSO

	CONCENTRATION		CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	DAY 3 (x1.0E+06)	SUSPENSION GROWTH	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE COLNING EFFICIENCY	RELATIVE TOTAL GROWTH
WITHOUT ACTIVATION										
	SOLVENT A		0.769	0.590	0.777	44.1	SOLVENT AVERAGE 46.9	#N/A	AVE. NO. COLONIES 206	
	SOLVENT B		0.797	0.604	0.825	49.6		206		
1	250	A	0.803	0.593	0.773	46.0	98%	241	117%	115%
2	250	B	0.757	0.653	0.771	47.6	101%	357	173%	175%
3	500	A	0.830	0.618	0.801	51.4	110%	149	72%	79%
4	500	B	0.840	0.587	0.822	50.7	108%	150	73%	79%
5	1000	A	0.764	0.618	0.884	52.2	111%	277	134%	149%
6	1000	B	0.807	0.598	0.757	45.7	97%	249	121%	117%
7	2500	A	0.734	0.546	0.596	29.9	64%	217	106%	68%
8	2500	B	0.743	0.594	0.665	36.7	78%	180	87%	68%
9	5000	A	0.497	0.403	0.604	15.1	32%	156	76%	24%
10	5000	B	0.529	0.425	0.648	18.2	39%	138	67%	26%

#N/A = Not Applicable

Suspension Growth (SG) = $\frac{\text{(Day 1 x Day 2 x Day 3) Cell Concentration}}{\text{2.0E+05 Cells/mL x (Day 1 x Day 2) Adjusted Cell Concentration}}$

Relative Suspension Growth (RSG) = $\frac{\text{SG of Treated Culture x 100}}{\text{Average SG of Solvent Controls}}$

Relative Cloning Efficiency = $\frac{\text{Average Viable Counts of Treated Culture x 100}}{\text{Average Viable Counts of Solvent Controls}}$

Total Growth = (RSG x RCE)/100

Table verified by: QAU: VL Study Director: QK

TABLE 14

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

EXPERIMENT NUMBER: B-2 (Confirmatroy Mutation Assay) TEST ARTICLE I.D.: 3-Nitro-1,2,4-Triazol-5-one (NTO)

MMS DOSE UNITS: µg/mL SITEK STUDY NUMBER: 0985-2400

SOLVENT: DMSO

CONCEN-TRATION	CELL CONCENTRATION DAY 1 (x1.0E+06)	DAY 2 (x1.0E+06)	DAY 3 (x1.0E+06)	SUSPEN-SION GROWTH	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
WITHOUT ACTIVATION					SOLVENT		AVE. NO.	
DMSO A	1.052	0.861	0.614	69.5	AVERAGE	203	COLONIES	
					65.4		182	
DMSO B	0.860	0.779	0.731	61.2		162		
MMS 15	0.878	0.237	0.148	3.9	6%	43	24%	1%
MMS 20	0.591	0.260	0.275	5.3	8%	1	1%	0%

MMS = Methyl Methanesulfonate

Suspension Growth (SG) = $\frac{\text{(Day 1 x Day 2 x Day 3) Cell Concentration}}{\text{2.0E+05 Cells/mL x (Day 1 x Day 2) Adjusted Cell Concentration}}$

Relative Suspension Growth (RSG) = $\frac{\text{SG of Treated Culture x 100}}{\text{Average SG of Solvent Controls}}$

Relative Cloning Efficiency = $\frac{\text{Average Viable Counts of Treated Culture x 100}}{\text{Average Viable Counts of Solvent Controls}}$

Total Growth = (RSG x RCE)/100

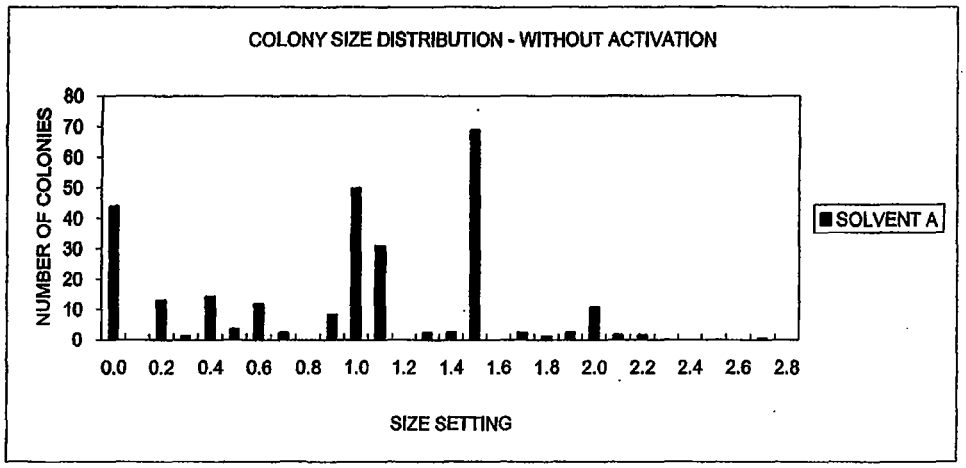
Table verified by: QAU: VL Study Director: BK

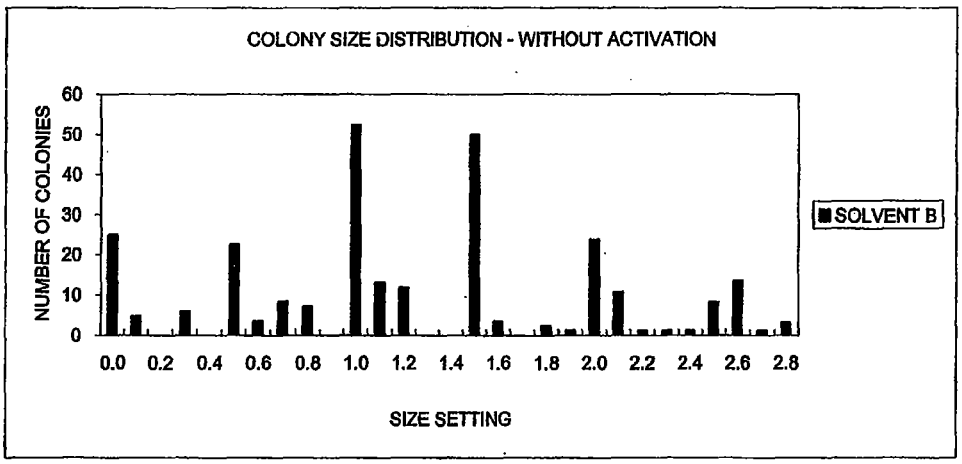
APPENDIX II

COLONY SIZING GRAPHS

Solvent Control Colony Size Histograms for the Definitive Assay without Activation

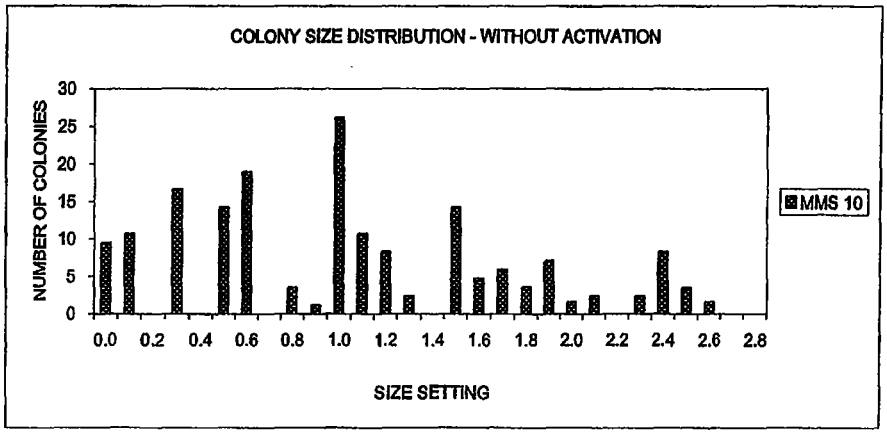
STUDY NUMBER: 0985-2400
EXPERIMENT NUMBER: B1
SOLVENT FOR TEST ARTICLE: DMSO

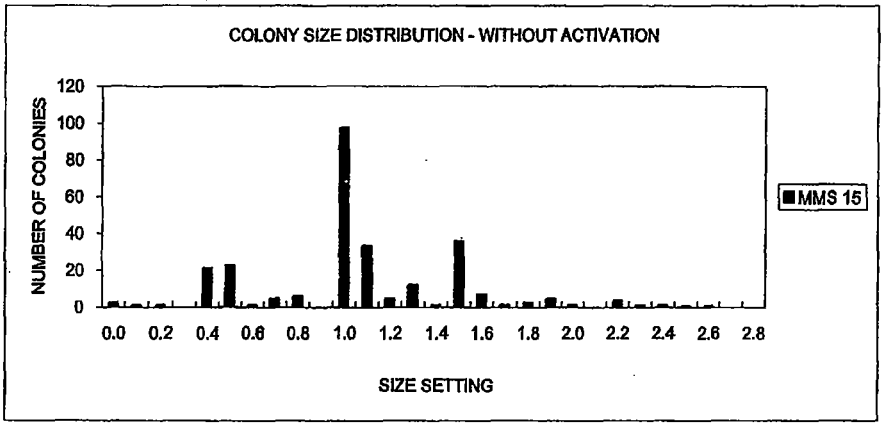


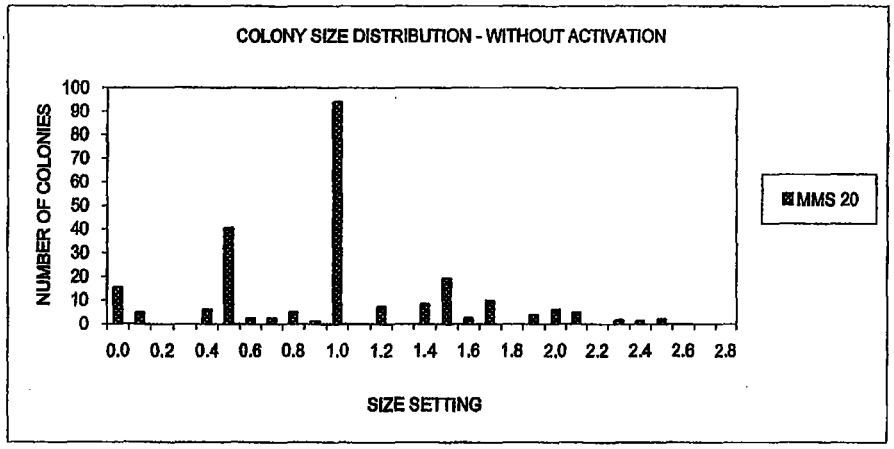


MMS Colony Size Histograms for the Definitive Assay without Activation

STUDY NUMBER: 0985-2400
EXPERIMENT NUMBER: B1
SOLVENT FOR MMS: WATER

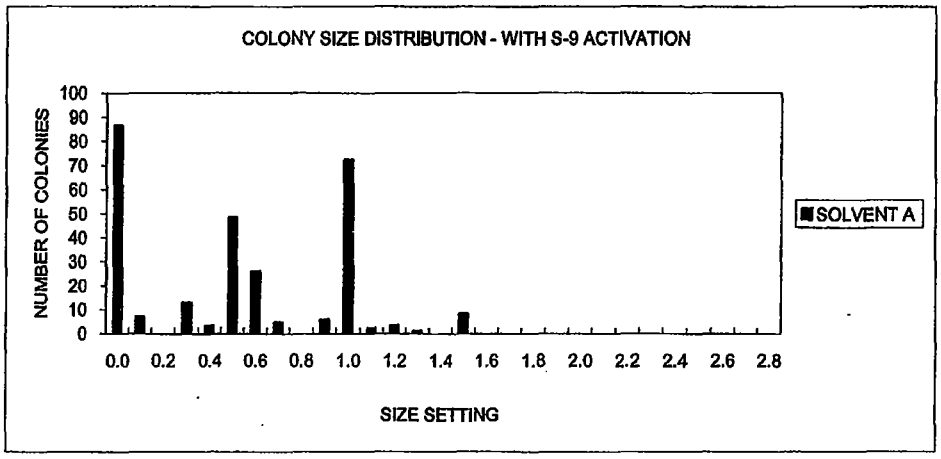


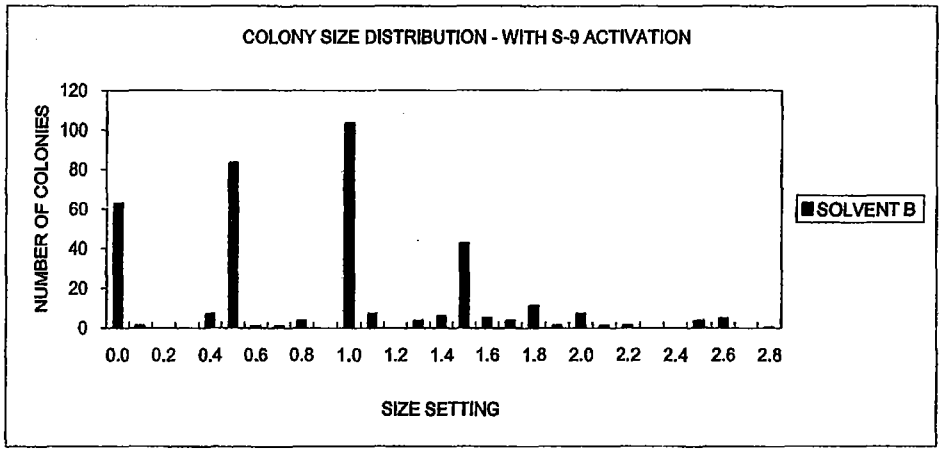




Solvent Control Colony Size Histograms for the Definitive Assay with Activation

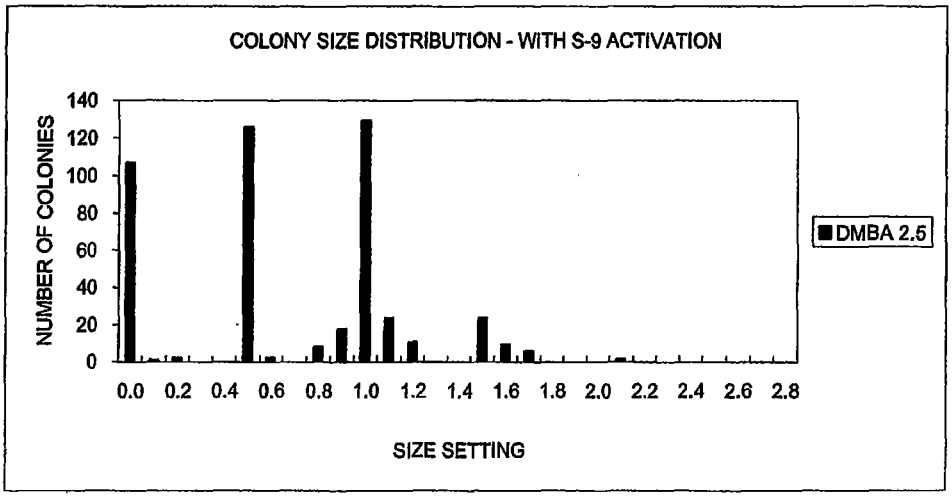
STUDY NUMBER: 0985-2400
EXPERIMENT NUMBER: B6
SOLVENT FOR TEST ARTICLE: DMSO

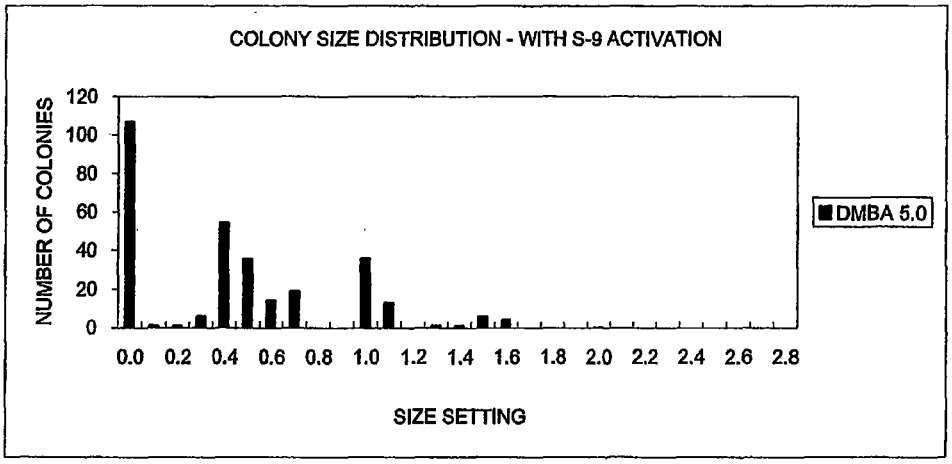




DMBA Colony Size Histograms for the Definitive Assay with Activation

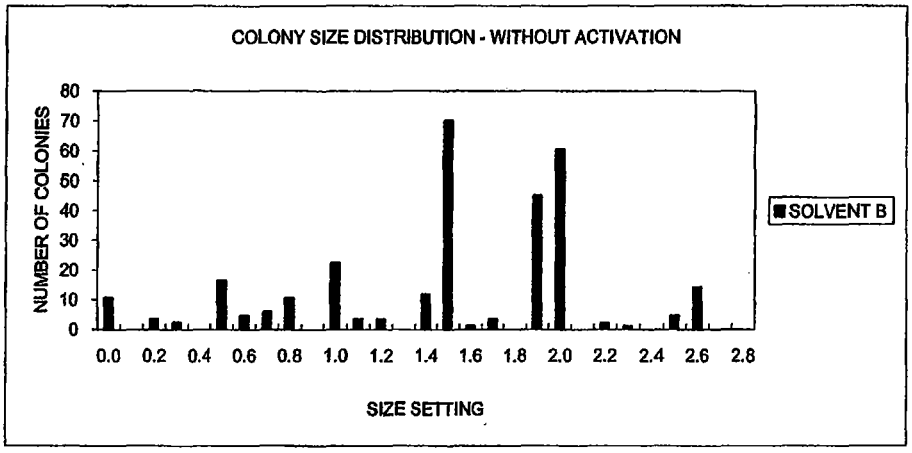
STUDY NUMBER: 0985-2400
EXPERIMENT NUMBER: B6
SOLVENT FOR DMBA: Acetone





Solvent Control Colony Size Histograms for the Confirmatory Assay without Activation

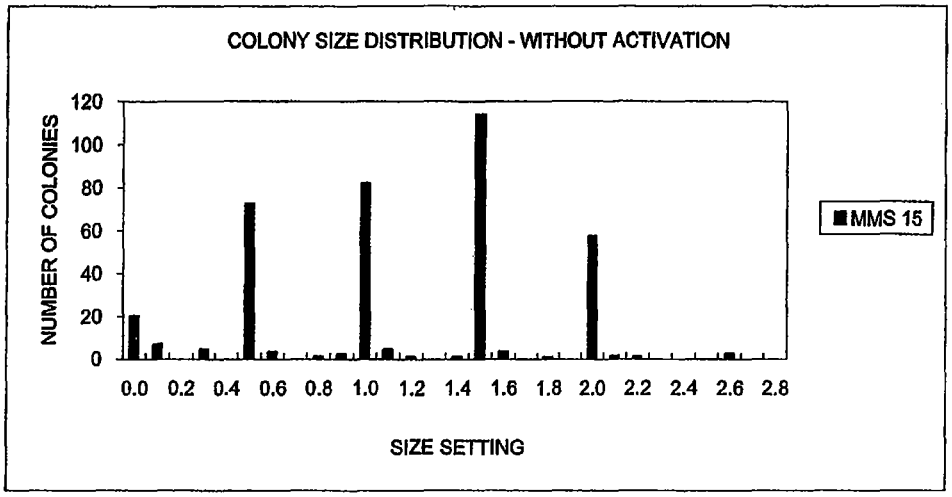
STUDY NUMBER: 0985-2400
EXPERIMENT NUMBER: B2
SOLVENT FOR TEST ARTICLE: DMSO

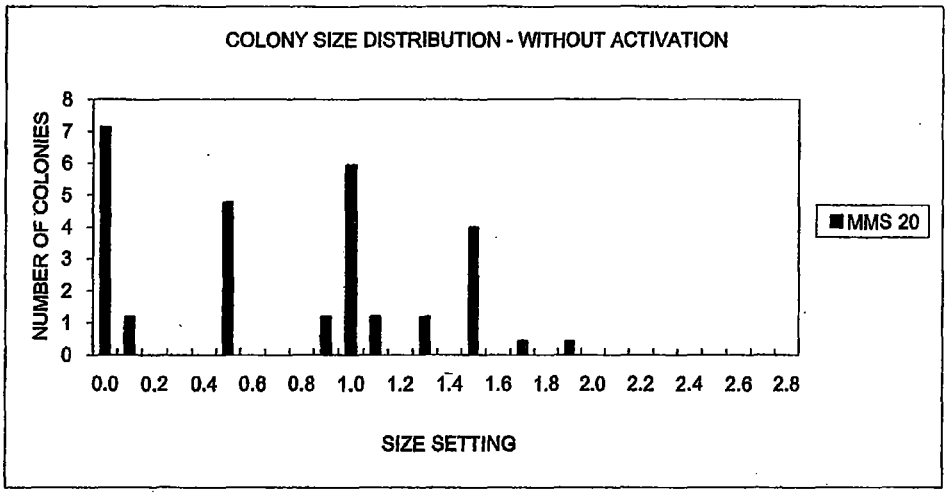


Solvent A was lost to contamination.

MMS Colony Size Histograms for the Confirmatory Assay without Activation

STUDY NUMBER: 0985-2400
EXPERIMENT NUMBER: B-2
SOLVENT FOR MMS: WATER





APPENDIX III

STUDY PROTOCOL, PROTOCOL AMENDMENTS AND PROTOCOL DEVIATIONS

EVALUATION OF A TEST ARTICLE IN THE L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY WITH COLONY SIZE EVALUATION IN THE PRESENCE AND ABSENCE OF INDUCED RAT LIVER S-9 WITH A CONFIRMATORY STUDY

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. **Please complete all bolded sections in Part One and sign section 8.0 to approve the protocol.**

PART ONE

1.0 SPONSOR

1.1 Name: US Army Center for Health Promotion and Preventive Medicine
Aberdeen Proving Ground, MD

1.2 Address: Aberdeen Proving Ground, MD 21010

1.3 Sponsor's Study Coordinator: Gunda Reddy, Ph.D., DABT

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

2.3 Study Director: Paul E. Kirby, Ph.D.

3.0 STUDY NUMBERS

* 3.1 Testing Facility's Study No.: 0985-2400

3.2 Sponsor's Study No.: Not Available

4.0 TEST ARTICLE

GLP's require that test article characterization information must be provided in the final report. This includes identification, lot number, purity, stability, source, and expiration date. As per regulatory requirements, lack of the above information will be cited as a GLP violation in the "Study Director's Compliance Statement" section of the final report.

* To be completed by the Testing Facility

4.1 Identification

Name: 3-Nitro-1,2,4-Triazol-5-one (NTO)

Batch/Lot No.: BAE 07B 305-001

4.2 Description

Color: White

Physical Form: Powder

4.3 Analysis

Purity Information: 99.6%

Does the Sponsor require the use of a correction factor to account for impurity?

____ Yes _X_ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

____ Room Temperature _X_ Refrigerated (1-5°C)

____ Frozen (-10 to -20°C)

____ Other (please specify): _____

Expiration Date: Not Available

4.5 Preferred Solvent (check one):

_X_ H_2O ___ Culture Medium ___ DMSO ___ Acetone ___ Ethanol

___ Other (please specify): _____

___ To be decided by the Testing Facility

4.6 **Special Handling Instructions:**

Use Standard Laboratory Safety Practices For Avoiding Exposure To Hazardous Substances.

5.0 REGULATORY AGENCY SUBMISSION

5.1 Test Design Specifications

This study protocol is designed to meet or exceed the EPA, OECD and ICH Guidelines specified in the following documents:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5300, Detection of gene mutations in somatic cells in culture. Revised July 1, 2002.

OECD Guideline for the Testing of Chemicals, No. 476. *In Vitro* Mammalian Cell Gene Mutation Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

5.2 Good Laboratory Practices

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792. Revised July 1, 2005.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58. Revised April 1, 2005.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45, [ENV/MC/CHEM(98)17], Paris 1998.

Will this study be submitted to a regulatory agency?

_X_ Yes ___ No

If so, which agency(ies)? Worldwide

6.0 DOSING SOLUTIONS

The Sponsor will be responsible for determining the strength and stability of the dosing solutions. The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113; EPA FIFRA = 40 CFR 160.113, and OECD GLPs, Section 6.2.

Does the Sponsor want dosing solution analysis?

___ Yes _X_ No**

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and/or stability of the dosing solutions. Stability will only be determined if the dosing solutions are not prepared immediately prior to each use. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and/or stability of the dosing solutions.

Dosing solution analysis will be performed by:

__ SITEK Research Laboratories __ Sponsor***

What dosing solutions will be analyzed?

** Additional charges will apply. See Special Services price schedule.

*** **Please note: All work pertaining to this study that is performed outside of SITEK is the responsibility of SITEK's Study Director. Therefore, as required by the GLPs, all of the following must be forwarded to the Study Director:**

- **All subcontract and/or Sponsor Quality Assurance audit findings and comments.**
- **Any deviations and/or amendments, if applicable.**
- **An original or copy of the analysis report.**
- **Location of where the raw data from the analysis will be archived.**

If the subcontract work is not performed under the GLPs, a statement by the Sponsor informing SITEK's Study Director of such must be provided.

From the Range Finding Test?

_____ Yes **_____ No**

From the Assay?

_____Yes **_____ No**

Which concentration(s)? _____

What amount of each concentration? _____

At what temperature should the dosing solutions be stored?

_____ Room Temperature **_____Frozen (-10 to -20°C)**

_____ Refrigerated (1-5°C)

At what temperature should the dosing solutions be shipped?

_____ Room Temperature **_____ On Wet Ice**

_____ On Dry Ice

7.0 STUDY DATES

* 7.1 Proposed Experimental Start Date: August 26, 2008

Defined as the date the cells are first treated with the test article in the Range Finding Test.

* 7.2 Anticipated Experimental Completion Date: October 24, 2008

Defined as the last date on which data are collected directly from the study.

* 7.3 Proposed Draft Report Date: November 14, 2008

7.4 Final Report: The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter.

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

8.0 PROTOCOL APPROVAL

* _____ 8-26-08
Study Director — Date

_____ 8-26-08
Sponsor's Study Coordinator — Date

* _____ 8-26-08
Quality Assurance Manager — Date

* _____ 8/26/08
Safety Officer — Date

* To be completed by the Testing Facility.

STUDY DESIGN

PART TWO

9.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause mutations at the thymidine kinase locus of L5178Y TK+/- mouse lymphoma cells.

10.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The L5178Y TK+/- Mouse Lymphoma Assay has been used extensively and has been demonstrated to be effective in detecting the mutagenic activity of chemicals from a wide range of classes.

11.0 ABBREVIATIONS

CM	-	Cloning Medium
DMBA	-	7,12-Dimethylbenz($^{\alpha}$)anthracene
DMSO	-	Dimethyl Sulfoxide
$RPMI_0$	-	RPMI Medium, no serum added, containing 0.1% pluronics and 0.011% sodium pyruvate
$RPMI_{10}$	-	$RPMI_0$ supplemented with 10% heat-inactivated horse serum
HIHS	-	Heat-Inactivated Horse Serum
IMF	-	Induced Mutant Frequency
MF	-	Mutant Frequency
MMS	-	Methyl methanesulfonate
NADP	-	Nicotinamide-adenine Dinucleotide Phosphate
RCE	-	Relative Cloning Efficiency
RSG	-	Relative Suspension Growth
SG	-	Suspension Growth
S-9	-	Induced Rat Liver Homogenate
TFT	-	Trifluorothymidine

TG	-	Total Growth
THG	-	Thymidine, Hypoxanthine and Glycine
THMG	-	Thymidine, Hypoxanthine, Methotrexate and Glycine
TK+/-	-	L5178Y cells heterozygous at the thymidine kinase locus
TK-/-	-	L5178Y cells homozygous recessive at the thymidine kinase locus
VC	-	Viable Count

12.0 INDICATOR CELLS

12.1 Source

The L5178Y TK+/- clone 3.7.2C mouse lymphoma cells were obtained from Jane Clarke in Biorelance, Rockville, Maryland on August 7, 2008. The cells were subcultured at SITEK Research Laboratories and cryopreserved in a large number of ampules.

12.2 Culture Conditions

The L5178Y TK+/- cells are routinely maintained in RPMI medium, supplemented with 10% heat-inactivated horse serum (HIHS) and pluronics. They are grown in an environment of 37 ± 1°C, approximately 5% CO_2 and 95% air.

12.3 Stock Cultures

The L5178Y TK+/- cells were propagated to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in RPMI Medium supplemented with 10% HIHS and 7-8% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Representative ampules were tested after cryopreservation for contaminating microorganisms, including mycoplasma. Stock ampules will be used to initiate the stock cultures for the test.

13.0 ROUTE OF ADMINISTRATION OF TEST ARTICLE

The test article will be administered in vitro directly or through a solvent compatible with the test cultures. This is the only route of administration available in this test system.

14.0 TEST SYSTEM IDENTIFICATION

All test cultures will be labeled with an indelible pen with a code system which clearly identifies the experiment number, the test article, controls, concentrations, and whether or not the culture was treated in conjunction with an exogenous activation system.

15.0 CONTROL SUBSTANCES

15.1 Positive Controls

Methyl methanesulfonate (MMS), which induces mutations in the absence of exogenous metabolic activation, will be used in the non-activated portion of the Assay. 7,12-Dimethyl-benz(α)anthracene (DMBA), which requires metabolic activation, will be used in the S-9 activated portion of the Assay.

The positive controls MMS and DMBA will be solubilized in deionized distill water and acetone respectively.

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

15.2 Vehicle Controls

The vehicles used for dissolving the test article and positive controls will be used as the vehicle controls. Deionized, distilled water, ethanol (CAS #64-17-5), acetone (CAS #67-64-1) and dimethyl sulfoxide (CAS #67-68-5) are some of the vehicles which are compatible with this test system. If there is a need to use other vehicles, the approval of the Sponsor will be obtained prior to their use. The amount of vehicle administered to the cell cultures will be limited to a level which has no detectable effect on the cultures.

16.0 DOCUMENTATION

All procedures, results, significant observations, and methods used for analysis of results will be documented in a study notebook. The study notebook will also include copies of the protocol, all protocol amendments and protocol deviations, study reports, and all relevant communications with the Sponsor.

17.0 EXPERIMENTAL PROCEDURE

17.1 Determination of Solubility/Miscibility

In order to determine the optimal vehicle for delivering the test article to the test system or to determine the maximum achievable concentration in the solvent requested by the Sponsor, a solubility/miscibility test will be performed. The solvents of choice for this system are water, DMSO, acetone and ethanol. If the test article is not sufficiently soluble in any of these solvents, additional solvents will be screened.

The solubility test will consist of weighing out 20-100 mg aliquots of test article and adding solvent in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved or until 5.0 mL of solvent has been added to the vessel. The volume of solvent required for complete dissolution and any additional observations will be recorded in the study notebook. Test articles that do not dissolve in 5.0 mL of solvent will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

17.2 Preparation of Test Cultures

The L5178Y TK+/- cells are routinely grown in RPMI medium with 10% HIHS, 0.011% sodium pyruvate and 0.1% pluronics. The complete medium will be designated as $RPMI_{10}$, and complete medium without horse serum will be designated as $RPMI_0$. Subcultures of L5178Y TK+/- mouse lymphoma cells will be periodically cleansed of spontaneously occurring TK-/- cells by growing the cells in $RPMI_{10}$ containing a mixture of thymidine, hypoxanthine, methotrexate and glycine (THMG). 3.0 mL of the THMG stock solution will be added to 100 mL cultures containing $0.1x10^6$ cells/mL. The cultures will then be gassed with approximately 5% CO_2 and 95% air and incubated at 37 ± 1°C. After an exposure period of approximately 24 hours, the THMG will be removed, and the cells will be grown for an additional period of approximately 24 hours in $RMPI_{10}$ containing thymidine, hypoxanthine and glycine (THG). Alternatively, pre-cleansed, cryopreserved L5178Y TK+/- cells may be reestablished and cultured for a short time (up to 2 weeks) prior to use.

Cultures of recently cleansed cells for use in the Range Finding Test or the Assay will be seeded in $RPMI_{10}$ at concentrations which will yield cell population densities in the range of $0.8x10^6$ to $1.6x10^6$ cells/mL at the time of use. Cultures (total volume of 6 mL) containing $1x10^6$ cells/mL will be prepared. These cultures contain 50% conditioned $RPMI_{10}$ (supernatant from the stock cultures) and 50% fresh $RPMI_0$.

17.3 Preparation of S-9 Metabolic Activation Mix

For the portion of the Assay in which the cells will be exposed to the test article in conjunction with an exogenous metabolic activation system, Aroclor or Phenobarbital and/or ß-Naphthoflavone- induced rat liver S-9 plus cofactors (S-9 mix) will be used as the activation system. For each mL of S-9 mix required, the following ratio of components will be prepared:

NADP	6.00 mg
DL-Isocitric Acid	11.25 mg
$RPMI_0$ Medium	0.75 mL
S-9 Homogenate	0.25 mL

The S-9 homogenate will be obtained from the induced livers of male Sprague-Dawley rats. It is prepared in 0.25M sucrose and kept frozen at less than -70°C for up to 3 years until it is thawed and used in the assay.

The aliquots of NADP and DL-isocitric acid will be mixed with the $RPMI_0$, and the pH of the resulting solution will be adjusted by adding 1N NaOH until the $RPMI_0$ returns to its original color. This solution will be filter sterilized by passage through a 0.45 µm filter. The S-9 homogenate will be added aseptically. This mixture will be diluted 1:1 with serum free medium and will be placed in an ice bath until used in the Assay.

17.4 Preparation of Test Article

The desired amount of the test article as specified in the dilution scheme will be weighed or measured just prior to use in either the Range Finding Test or the Assay. The dosing solutions will be prepared by adding the appropriate volume of solvent to the test article and thoroughly mixing the resulting solution until the test article goes completely into solution or a

homogeneous suspension is achieved. The remaining doses specified in the dilution scheme will be prepared by either performing a serial dilution or by varying the volume delivered from the stock concentration to the cultures. In all treatments the amount of solvent delivered to the target cultures will be limited to a level which has no cytotoxic effect on the cells. If necessary, the test article may be added directly to the culture medium.

17.5 Range Finding Test

In order to determine the test article concentrations that will produce from 0-100% cytotoxicity, a Range Finding Test will be performed. The test article will be weighed and a serial dilution prepared. Unless there are solubility/miscibility limitations, prior knowledge of cytotoxicity indicates differently, or the Sponsor specifies differently, the treatment concentrations for the test article will be 5000, 1000, 500, 100, 50, 10, 5.0, 1.0, 0.5 and 0.1 µg/mL. If the results based on the dosing regimen indicate that the threshold level of complete toxicity will be below 0.1 µg/mL, an additional Range Finding Test will be performed. In the instances where the test article forms precipitate, the lowest precipitating concentration will be used as the highest dose.

17.5.1 Treatment

Treatment will be performed by adding 4.0 mL of $RPMI_0$ or 4.0 mL of S-9 mix to the culture and then adding the appropriate volume of test article/vehicle mixture. Two solvent control cultures will run simultaneously with the treated cultures. The cultures will be gassed with approximately 5% CO_2 and 95% air and incubated at 37 ± 1°C on a roller drum apparatus rotating at 25 ± 2 rpm. After a 4-hour exposure period, the cells will be pelleted by centrifuging them at approximately 1000 rpm for 10 minutes, and the test article will be removed by pouring off the supernatant. Two rinses in 10 mL of $RPMI_{10}$ will be performed, after which the cells will be resuspended in 30 mL of $RPMI_{10}$, gassed with approximately 5% CO_2 and 95% air, and incubated at 37 ± 1°C on a roller drum rotating at 25 ± 2 rpm.

17.5.2 Determination of Cytotoxicity

At approximately 20 hours and 44 hours post treatment, samples will be removed from each culture to determine the cell population density of each. Each sample will be placed in a vial containing 0.1% trypsin. The vials will be incubated for 10 minutes at 37 ± 1°C, after which they will be placed on an automatic cell counter. Three coincidence-corrected counts will be made, and the average count will be used to determine the concentration of cells per mL for each culture. After the determination of cell numbers at 20 hours post treatment, each culture having greater than $0.2x10^6$ cells/mL will be adjusted to $0.2x10^6$ cells/mL. This will be performed by retaining the volume of culture that will result in a final concentration of $0.2x10^6$ cells/mL when fresh medium is added to it to yield a combined final volume of 30 mL.

The Suspension Growth (SG) of each culture will be determined using the following formula:

$$SG = \frac{\text{Day 1 Cell Conc.}}{0.2x10^6 \text{ Cells/mL}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

The Relative Suspension Growth (RSG) of each of the test article-treated cultures will be determined by calculating its growth relative to the corresponding solvent control cultures' average SG.

$$RSG = \frac{\text{SG of Treated Culture}}{\text{Average SG of Solvent Controls}} \times 100$$

17.6 Mutation Assay

Based on the results of the Range Finding Test(s), doses for use in the Assay will be selected to produce a range of concentrations that produce from 10-100% RSG. The selection of the range between doses and whether or not single cultures or replicate cultures per dose will be treated will be based on the steepness of the toxic response observed in the Range Finding Test(s). Those test articles having very steep toxic response curves will be tested over a narrow range with duplicate cultures per dose. Test articles having broad toxic response curves will be tested over a wide range of doses with single cultures per dose. At least 16 cultures plus two solvent control cultures will be included in each treatment group, i.e., with activation and without activation. The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment. In addition, two sets of cultures will be treated with positive control chemicals. MMS will be the positive control chemical for the non-activated portion of the Assay, and DMBA will be the positive control chemical for the S-9 activated portion of the Assay.

The pH of the test article treated cultures will also be monitored and adjusted to the normal pH for the system by addition of 1N HCl or 1N NaOH.

17.6.1 Test Culture Preparation and Exposure

The test article will be weighed and a serial dilution performed as previously described in Section 17.4. Cultures containing 6.0 mL of cells at a concentration of $1x10^6$ cells/mL will be prepared. These cultures will contain 50% conditioned $RPMI_{10}$ (supernatant from the stock cultures) and 50% fresh $RPMI_{10}$. Immediately prior to adding the appropriate aliquots of the test article dosing solution, 4.0 mL of either $RPMI_{10}$ medium or 4.0 mL of S-9 mix will be added to each culture depending on whether or not it is to be treated in conjunction with the exogenous metabolic activation system. Immediately after addition of the test article, the culture will be gassed with approximately 5% CO_2 and 95% air. The cultures will be placed on a roller drum apparatus and rotated at a speed of 25 ± 2 rpm in an environment of 37 ± 1°C.

17.6.2 Termination of Treatment

After a 4-hour exposure period, the cells will be pelleted by centrifugation at approximately 1000 rpm for 10 minutes, and the test article will be removed by pouring off the supernatant. Two rinses in 10 mL of $RPMI_{10}$ will be performed, followed by resuspension in 30 mL of $RPMI_{10}$, gassing with approximately 5% CO_2 and 95% air, and incubation at 37 ± 1°C on a roller drum apparatus set at 25 ± 2 rpm.

17.6.3 Maintenance of Test Cultures During the Expression Period

At approximately 20 hours and 44 hours post treatment, samples will be removed from each culture to determine the cell population density of each. Each sample will be placed in a vial containing 0.1% trypsin. The vials will be incubated for 10 minutes at 37 ± 1°C, after which they will be placed on an automatic cell counter. The average of three coincidence-corrected counts will be used to determine the concentration of cells per mL for each culture. After the determination of cell numbers, each culture having a population greater than $0.2x10^6$ cells/mL will be adjusted to $0.2x10^6$ cells/mL. At the 20-hour point the final volume after adjustment will be 30 mL. For the 44-hour point the final volume will be 15 mL.

17.6.4 Cloning for Mutant Selection and Viability Determination

After the 2-day expression period, cultures will be selected for cloning based on their SG. Only cultures having a population density of $0.2x10^6$ cells/mL or greater will be clonable. As many as 12 cultures per treatment group may be cloned depending on the toxic response curve.

For each culture selected for cloning, 200 mL of cloning medium (CM) will be prepared. The CM will be made by combining the following ingredients in the indicated proportions for each 100 mL of CM.

$RPMI_0$	70.75 mL
Horse Serum	20.0 mL
Sodium Pyruvate (0.022 gm/mL)	0.5 mL
Purified Agar (4% Solution)	8.75 mL

For each culture selected for cloning, 100 mL of CM will be dispensed into a flask designated for the addition of the restrictive agent trifluorothymidine (TFT), which permits the growth of TK-/- cells only, and 100 mL will be dispensed into a flask designated as a Viable Count (VC) flask. The CM in the VC flask will be used to culture an aliquot of cells from each culture cloned to approximate the percentage of viable cells in each culture.

The cloning process will be as follows:

1. Each TFT and VC flask will receive 100 mL of CM, and each will be placed in a shaker incubator set at approximately 125 rpm and 37 ± 1°C.

2. The cultures designated for cloning will be centrifuged at a speed of approximately 1000 rpm for 10 minutes. 12 mL of the supernatant will be aspirated and discarded. The cells will be resuspended in the remaining volume of supernatant and then added to the appropriate TFT flask. Each TFT flask will contain $3x10^6$ cells. Each flask will be replaced on the shaker incubator (125 rpm, 37 ± 1°C).

3. After each TFT flask has shaken for at least 15 minutes, a 1.0 mL aliquot will be removed from each, a 1:10 and 1:5 serial dilution will be performed, and a 1.0 mL aliquot of the last dilution will be added to the appropriate VC flask. Each VC flask will contain approximately 600 cells.

4. After the completion of cell addition to each VC flask, an aliquot of TFT will be added to each TFT flask. The concentration of TFT in the culture will be approximately 3.0 µg/mL. Both TFT and VC flasks will be replaced on the shaker incubator (125 ± 2 rpm, 37 ± 1°C).

5. After at least 15 minutes of mixing, the contents of each flask will be dispensed in equal aliquots onto three plates. The plates will be chilled for 20 minutes in a refrigerator (1-5°C) and then placed in an incubator at 37 ± 1°C in an atmosphere of approximately 5% CO_2 and 95% air.

6. 3-4 days after cloning, the plates will be carefully observed for signs of contamination. Any contaminated plates will be discarded, and the identity of these plates will be recorded in the study notebook.

17.6.5 Enumeration of Colonies

After completion of the 10- to 12-day incubation period, the number of colonies per TFT and VC plate will be determined. The colony numbers and sizes will be determined with an ARTEK 880 Colony Counter equipped with a ten-turn potentiometer.

Each plate will be placed on the stage and three counts will be made with the automatic counter. The plate will be rotated on the stage approximately 120^0 between each count and the median count will be recorded. For sizing purposes, the diameters of colonies from the positive controls, positive test article doses and the corresponding solvent controls will be measured. The smallest colonies detectable by the counter, which are approximately 0.1 to 0.2 mm, will be counted, and then the number of colonies in each group increased in size by approximately 0.1 mm will be determined until all colonies have been sized. The number of colonies in a particular size range will be determined by subtracting the number of colonies counted in the next higher size range.

Each median count will be corrected as specified in SITEK's SOP's, Section 24.11.4, to give the closest approximation of the actual number of colonies per plate.

17.6.6 Determination of Mutant Frequency and Induced Mutant Frequency

The Mutant Frequency (MF) of each culture that is successfully cloned will be determined as a function of viable cells forming colonies. The calculation will be performed as follows:

$$\text{MF Per } 10^6 \text{ Viable Cells} = \frac{\text{Average No. of Mutants Per Plate}}{\text{Average No. of Colonies in the Corresponding VC plates}} \times 200$$

The Induced Mutant Frequency (IMF) will be calculated by using the following formula:

$$\text{IMF} = \text{MF of Treated Cultures} - \text{Average MF of Solvent Control Cultures}$$

17.6.7 Determination of Relative Suspension Growth, Relative Cloning Efficiency and Total Growth

17.6.7.1 Relative Suspension Growth

The SG and RSG of each culture will be determined by performing the calculations described earlier in Section 17.5.2.

17.6.7.2 Relative Cloning Efficiency

The Relative Cloning Efficiency (RCE) will be determined for each culture by using the following formula:

$$RCE = \frac{\text{Average VC Count of Treated Culture}}{\text{Average VC Count of Solvent Controls}} \times 100$$

17.6.7.3 Total Growth

The Total Growth (TG) of a culture is a figure which encompasses both the RSG and the RCE. It will be calculated as follows:

$$TG = \frac{RSG \times RCE}{100}$$

$$RTG = \frac{\text{TG of Treated Culture}}{\text{Average TG of Solvent Controls}} \times 100$$

The TG will be calculated for each test article-treated culture that is successfully cloned.

17.6.8 Preparation of Colony Size Histograms

A histogram of the colony size distribution will be prepared for the mutant colonies appearing in the restrictive medium plates. The number of colonies in each size range will be plotted for any positive test article-treated cultures and positive and negative controls.

17.7 Confirmatory Mutation Assay

If the first mutation assay produces negative results, a confirmatory mutation assay will be performed in which the exposure period without activation is extended to 24 hours. The activated portion of the assay may also be repeated using the same exposure period as in the first assay. The experimental procedure for the confirmatory assay will be altered in the following manner:

1. The exposure period without activation will be 24 hours.

2. $2x10^5$ cells/mL will be treated in 30 mL of treatment medium.

3. The final serum concentration in the treatment medium will be 6.7%.

4. Cells will be readjusted at 24, 48 and 72 hours. 24- and 48-hour readjustments will be in 30 mL of culture medium, however, the 72-hour readjustment will be in 15 mL of culture medium.

5. The test article concentrations will be adjusted to allow for greater toxicity because of the extended exposure period.

6. Other parameters for both the non-activated and activated portions of the assay may be altered to increase the sensitivity of the test.

17.8 Criteria For a Valid Assay

The following criteria must be met for an Assay to be considered acceptable.

17.8.1 Solvent Control Cultures

1. The average Cloning Efficiency of the solvent control cultures must be 65% or higher.

2. The average MF of the solvent control cultures must be between 35 to 140 per 10^6 viable cells.

3. The suspension growth for the solvent controls must be between 8 and 32.

17.8.2 Positive Controls

The results for the positive control cultures will be considered acceptable if:
1. At least one of the positive control treated cultures has a MF that is three times or greater than the average MF of its solvent control cultures.

2. Their solvent controls have an average Cloning Efficiency of 65% or greater.

17.9 Evaluation of Test Results

The following criteria will be used as guidelines in evaluating the results of the Assay for a negative, positive or equivocal response. Since it will be impossible to write criteria that would apply to every configuration of data generated by the Assay, the Study Director will be responsible for the ultimate decision in the evaluation of the results. The factors considered in making the decision will be discussed in the report.

17.9.1 Criteria for a Negative Response

A response will be considered negative if,

1. All of the cultures exhibiting a TG of approximately 10% and greater have MF's that are less than twice that of the mean MF of the corresponding solvent control cultures, and

2. There is no evidence of a dose-dependent response.

17.9.2 Criteria for a Positive Response

A response will be considered positive if at least one dose has a MF that is two times or more greater than the average MF of the corresponding solvent control cultures and the response is dose dependent. In evaluating the results, consideration will be given to the degree of toxicity exhibited by the culture having the twofold or greater increase in MF and the magnitude of the increase in MF.

17.9.3 Criteria for an Equivocal Response

A response will be considered equivocal if it does not fulfill the criteria of either a negative or a positive response, and/or the Study Director does not consider the response to be either positive or negative.

18.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor. These changes and the reasons for them will be documented and attached to the protocol as an addendum. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and as soon as practical, such changes along with their reasons or explanations will be documented and kept in the study notebook.

19.0 REPORT OF RESULTS

19.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter. The report will include, but not be limited to, the following:

1. Name and address of the testing facility and the dates on which the study was initiated and completed, terminated or discontinued.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Methods used to analyze the data.

4. The test and control substances.

5. Description of the methods used to perform the study.

6. The name and signature of the Study Director and the names of other technical personnel who participated in performing the study.
7. The location where the raw data and reports are to be stored.

8. A statement from the Quality Assurance Unit.

19.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments which will include the reason(s) for the change, and these amendments will be added to the final report as an addendum.

20.0 ARCHIVES

The raw data, documentation, protocol and Final Report, along with an electronic file containing the data tables and copy of the Final Report of the study will be maintained in SITEK Research Laboratories' Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland, for ten years.

21.0 REFERENCES

1. Clive, D., and J. Spector. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mut. Res., 31:17-29, 1975.

2. Clive, D., K. O. Johnson, J. F. S. Spector, A. G. Batson, and M. M. M. Brown. Validation and Characterization of the L5178Y/TK+/- Mouse Lymphoma Mutagen Assay System. Mut. Res., 59:61-108, 1979.

3. Moore-Brown, M. M. , D. Clive, B. E. Howard, A. G. Batson, and K. O. Johnson. The Utilization of Trifluorothymidine (TFT) to select for Thymidine Kinase deficient (TK-/-) Mutants from L5178Y/TK+/- Mouse Lymphoma Cells. Mut. Res., 85:363-378, 1981.

4. Kirby, P. Mouse Lymphoma Cell Assays, in: J.F. Douglas (ed.), Carcinogenesis and Mutagenesis Testing, Humana Press, Clifton, N.J., pp. 207-226, 1984.

5. Moore, M. M., D. Clive, B. E. Howard, A. G. Batson, and N. T. Turner. In Situ Analysis of Trifluorothymidine-resistant (TFT^R) mutants of L5178Y/TK+/- Mouse Lymphoma Cells. Mut. Res., 151:147-159, 1985.

6. Clive, D., W. Caspary, P. E. Kirby, R. Krehl, M. Moore, J. Mayo, and T. J. Oberly. Guide for Performing the Mouse Lymphoma Assay for Mammalian Cell Mutagenicity. Mut. Res., 189:143-156, 1987.

PROTOCOL AMENDMENT 1

Amendment No.:	1
Sponsor:	US Army Center for Health Promotion and Preventive Medicine Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-2400
Sponsor's Study No.:	N/A
Test Article I.D.:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Evaluation of a Test Article in the L5178Y TK +/- Mouse Lymphoma Mutagenesis Assay with Colony Size Evaluation in the Presence and Absence of Induced Rat Liver S-9 with a Confirmatory Study
Amendment No. 1:	Protocol page 2, section 4.5: Preferred solvent was changed from H_2O to DMSO.
Reason for Amendment No. 1:	The test article was not sufficiently soluble in H_2O.

APPROVED:

Paul E. Kirby, Ph.D.
Study Director

8-28-08
Date

PROTOCOL AMENDMENT

Amendment No.:	2
Sponsor:	US Army Center for Health Promotion and Preventive Medicine
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-2400
Sponsor's Study No.:	Not Available
Test Article ID:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Evaluation of a Test Article in the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay with Colony Size Evaluation in the Presence and Absence of Induced Rat Liver S-9 with a Confirmatory Study

Amendment No. 2: Protocol Page 12, Section 17.6, Mutation Assay, The Definitive and Confirmatory Mutation Assays were performed with and without activation at concentrations of 10, 50, 100, 250, 500, 1000, 2500 and 5000 µg/mL.

Reason for Amendment No. 2: As stated in the protocol, the test article concentrations will be included in the form of an amendment.

APPROVED:

Paul E. Kirby, Ph.D.
Study Director

14-5-08
Date

PROTOCOL DEVIATIONS

Deviation Nos.:	1 and 2
Sponsor:	US Army Center for Health Promotion and Preventive Medicine
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0985-2400
Sponsor's Study No.:	Not Available
Test Article ID:	3-Nitro-1,2,4-Triazol-5-one (NTO)
Protocol Title:	Evaluation of a Test Article in the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay with Colony Size Evaluation in the Presence and Absence of Induced Rat Liver S-9 with a Confirmatory Study

Deviation No. 1: Protocol Page 16, Section 17.8.1, Solvent Control Cultures, Item 2 indicates that the Mutant Frequency of the solvent control cultures should be between 35 and 140 per 10^6 viable cells. The average mutant frequency for the solvent controls in the Definitive Mutation Assay with activation was 144.

Reason for Deviation No. 1: Solvent A had a higher than normal Mutant Frequency because of a low cloning efficiency. This had no affect on the outcome of the study as the Mutant Frequencies of the treated cultures had no indication of a test article induced increase in Mutant Frequencies.

Deviation No. 2: The cell cultures prepared for treatment in the Range Finding test contained 6.7% horse serum instead of 5% horse serum.

Reason for Deviation No. 2.: Calculation error. This error did not affect the validity of the Range Finding Test.

APPROVED:

Paul E. Kirby, Ph.D.
Study Director

11-7-08
Date

APPENDIX IV

HISTORICAL SOLVENT AND POSITIVE CONTROL DATA

SITEK RESEARCH LABORATORIES

L5178YTK +/- MOUSE LYMPHOMA MUTATION ASSAYS

HISTORICAL SOLVENT CONTROLS DATA

WITHOUT S-9 ACTIVATION

	DMSO	SALINE	PBS	WATER	CULTURE MEDIUM	ETHANOL	ACETONE
Average	74	73	93	59	102	35	59
Standard Deviation	25	6	10	15	25	5	8
Minimum Value	0	69	86	32	78	30	48
Maximum Value	146	77	100	94	150	39	69
N*	103	2	2	16	4	2	8

WITH S-9 ACTIVATION

	DMSO	SALINE	PBS	WATER	CULTURE MEDIUM	ETHANOL	ACETONE
Average	72	76	104	62	106	57	76
Standard Deviation	21			22	45	8	21
Minimum Value	31	76	104	34	69	49	29
Maximum Value	115	76	104	99	157	64	125
N*	39	1	1	14	3	2	71

N* = Number of data points

SITEK RESEARCH LABORATORIES

L5178YTK +/- MOUSE LYMPHOMA MUTATION ASSAYS

HISTORICAL DATA FOR POSITIVE CONTROLS

Hycanthone	0.5 ug/mL	1.0 ug/mL	5.0 ug/mL	10 ug/mL
Average	307	507	352	479
Standard Deviation	192	278	223	246
Minimum Value	96	143	86	134
Maximum Value	1148	1641	1113	1156
N*	14	15	36	34

DMBA**	1.25 ug/mL	2.5 ug/mL	5 ug/mL	7.5 ug/mL
Average	142	239	282	237
Standard Deviation	89	221	156	403
Minimum Value	79	33	93	52
Maximum Value	205	1185	724	1737
N*	2	26	39	15

* = Number of data points
** = 7,12-Dimethylbenz(a)anthracene

Note: Historical data for Methyl methanesulfonate (MMS) is not yet available. SITEK Research Laboratories has just begun using MMS as a positive control for the Mouse Lymphoma Assay because Hycanthone is no longer available.

APPENDIX V

S-9 BATCH INFORMATION

MOLTOX POST MITOCHONDRIAL SUPERNATANT (S-9) PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2188 **SPECIES:** Rat **PREPARATION DATE:** October 3, 2007
PART NO.: 11-111 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** October 3, 2009
VOLUME: 2ml **SEX:** Male **BUFFER:** 0.25 M Sucrose
TISSUE: Liver **INDUCING AGENT(s):** Aroclor 1254
REFERENCE: Maron, D & Ames, B. *Mutat. Res.* 113:173, 1983 Monsanto Lot No. KL615 - 500mg/kg

BIOCHEMISTRY:

- PROTEIN

35.8 mg/ml — Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	89.2
PROD	2B1, 3B2	23.7
MROD	1A2	151.9
BROD	3A, 2B	90.0

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-and benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, MROD) were conducted using a modification of the methods of Burke et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions calculated as the ratio of the sample vs. uninduced control activities (SA). Control SA's (pmoles/min/mg protein) for EROD, PROD, BROD, & MROD were 20.6, 10.5, 26.6, & 5.7 respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants EtBr/	CPA/
TA98	TA1535
200.4	830

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.***113**:173, 1983).

	µl S9 per plate/number his$^+$ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5.0 µg)	110	185	426	663	859	1045
2-AA (2.5 µg)	130	546	1455	1808	1823	1463

MOLECULAR TOXICOLOGY, INC.
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Boone, NC 28607
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MOLTOX POST MITOCHONDRIAL SUPERNATANT (S-9) PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2220 **SPECIES:** Rat **PREPARATION DATE:** December 5, 2007
PART NO.: 11-111 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** December 5, 2009
VOLUME: 2ml **SEX:** Male **BUFFER:** 0.25 M Sucrose
TISSUE: Liver **INDUCING AGENT(s):** Aroclor 1254
REFERENCE: Maron, D & Ames, B. *Mutat. Res.* 113:173, 1983 Monsanto Lot No. KL615 - 500mg/kg

BIOCHEMISTRY:

- PROTEIN

40.1 mg/ml

Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	101.0
PROD	2B1, 3B2	24.7
BROD	3A, 2B	22.6
MROD	1A2	50.0

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-and benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, MROD) were conducted using a modification of the methods of Burke et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions calculated as the ratio of the sample vs. uninduced control specific activities (SA). Control SA's (pmoles/min/mg protein) were 30.9, 17.2, 54.2, & 10.4 for EROD, PROD, BROD, & MROD respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants EtBr/ TA98	CPA/ TA1535
296.4	600

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.***113**:173, 1983).

	µl S9 per plate/number his$^+$ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5.0 µg)	105	142	396	761	1002	1048
2-AA (2.5 µg)	95	449	1219	1452	1721	1615

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APPENDIX VI

CERTIFICATE OF ANALYSIS

[redacted]

Ordnance Systems Kingsport, Tennessee

Certificate of Analysis for: NTO Per ORDNANCE SYSTEMS SPECIFICATION

Certificate No.: BAE 1686
Customer PO No.: 2007003323 **Customer Part No.:** N/A

	Batch Number:	10NTO7-3
	Containers Shipped:	14
	Lot Number:	BAE07B305-001
Characteristics	Specs	
% Purity by HPLC	99.0 - 100.0	99.6
Acidity, % as Nitric	0.01 Max	0.00
Exotherm Onset, Deg. C	250 Min	265
% Moisture	0.05 Max	0.03
Impact,cm		52
Appearance, Crystalline Solid	White to Pale Yellow	Pale Yellow Crystalline
Workmanship	PASS	PASS

Total Drums: 14 **Total Pounds:** 800

77

Prepared by: Neal Roberts

Digitally signed by Neal Roberts
DN: cn=Neal Roberts, c=US, o=BAE Systems, ou=Quality Assurance, email=neal.j.roberts@baesystems.com
Date: 2008.08.04 16:48:08 -04'00'

Neal Roberts, Quality Manager
Wednesday, February 28, 2007

[redacted]

Destination Control:
These items are covered by the United States Munitions List (USML) and is therefore subject to control by the U.S. Department of State. Export requires an export license, or other approval, issued by the U.S. Department of State.

Study No. 0985-2400